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CHARACTERIZATION AND EXPRESSION OF RAT INTESTINAL L-LYSINE TRANSPORT SYSTEMS

BY

WILLIAM R. MUZYKA ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

DEPARTMENT OF PHYSIOLOGY

Edmonton, Alberta
Fall, 1994

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **CHARACTERIZATION AND EXPRESSION OF RAT INTESTINAL L-LYSINE TRANSPORT SYSTEMS** submitted by **WILLIAM R. MUZYKA** in partial fulfillment of the requirements for the degree **MASTER OF SCIENCE**.

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This thesis is dedicated to my parents Mary and Russell, my sister Stephanie and husband Tim, all of whom supported me throughout, and to the love of my life, Marissa, who encouraged and helped me through the tough times and actually understood what I endured for three long years.

ABSTRACT

L-lysine transport across the intestinal brush-border membrane (BBM) of rat was studied using the everted sleeve technique. The uptake of lysine into enterocytes occured through high affinity Na^+-independent (71%) and Na^+-dependent (29%) routes, both of which were completely inhibited by 5 mM L-arginine in the presence and absence of Na^+. L-leucine inhibited both the Na^+-independent and Na^+-dependent components by 67% in both NaCl and ChCl media. Other neutral amino acids, L-alanine, L-phenylalanine, and L-valine, were relatively weak inhibitors of BBM lysine transport. These results suggest multiple systems for lysine transport across the intestinal BBM.

The *Xenopus* oocyte expression system was employed to further characterize and identify intestinal lysine transporters. Injection of rat intestinal mRNA into oocytes resulted in a 4-5 fold increase in lysine transport compared with water-injected controls. This expressed lysine transport activity had both Na^+-independent (60%) and Na^+-dependent (40%) components and was completely inhibited by 5 mM L-arginine both in the presence and in the absence of Na^+, thus indicating transport not diffusion. Kinetic analysis showed the expression of high affinity lysine transport: apparent K_m - 0.17 mM (NaCl) and 0.22 mM (ChCl). In the presence of Na^+ complete inhibition was observed with L-leucine, but with the removal of Na^+, leucine's ability to inhibit was substantially reduced to 67% of the Na^+-independent component. No other amino acids tested produced significant inhibition of lysine uptake. The results suggested the expression of multiple intestinal L-lysine transport systems.

The size of the intestinal mRNAs encoding lysine transporters was determined through size fractionation. A peak fraction (1.5-2.25 kb) was identified for exogenous lysine transport activity. A cDNA library was constructed and screened using the *Xenopus* oocyte expression system, resulting in the isolation of a cDNA encoding rat intestinal 4F2hc glycoprotein. Injection of cRNA from the 4F2hc clone induced a 3-4 fold increase in lysine uptake over background, with a K_m values for lysine transport of 46 μM and 44 μM in the presence and absence of Na^+, respectively. L-leucine inhibition of lysine transport was complete in the presence of Na^+ and was substantially reduced in the absence of Na^+. Efflux experiments demonstrated *trans*-stimulation of lysine efflux by leucine in the presence of Na^+ in oocytes injected with the 4F2hc cRNA. Hydropathy analysis suggested the rat 4F2hc protein contained a single transmembrane domain.

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LIST OF ABBREVIATIONS

4F2hc	Murine heavy chain antigen
1 A_{260}	1 Absorbance unit at 260 nm
ALA	L-alanine
ARG	L-arginine
BBM	Brush border membrane
BLM	Basolateral membrane
cDNA	Complementary deoxyribonucleic acid
ChCl	Choline chloride
CONT	Control uptake or influx
cRNA	Complimentary ribonucleic acid
d	Density
dpm	Disintegrations per minute of radioactivity
ecoR1	Ecotropic murine virus receptor
EDTA	Ethylenediamine tetraacetic acid
g	acceleration due to gravity
gm	gram
hr	hour
IC_{50}	Half-maximal Inhibition
K_m	Carrier-subtrate affinity

LB	Luria-Bertini culture medium
LEU	Leucine
LPI	Lysinuric protein intolerance
MBM	Modified Barth's medium
M.Q. Water	Milli-pore filtered distilled water
mCAT	Mouse cationic amino acid transporter
min	Minute
mRNA	Messenger ribonucleic acid
nl	nanolitre (10^{-9} litres)
nmol	nanomole (10^{-9} moles)
ng	nanogram (10^{-9} grams)
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
pH	Negative log of hydrogen ion concentration
PHE	L-phenylalanine
pmol	picomole (10^{-12} moles)
pg	picogram (10^{-12} grams)
rBAT	rabbit basic amino acid transporter
R.P.M.	Revolutions per minute
SA-PMP	Streptavidin Magnesphere Particles
SDS	Sodium dodecyl sulfate

SEC.	Section
S.E.M.	Standard error of the mean
S.E.D.M.	Standard error of the difference between the means
SLS	Sodium lauryl sarcosinate
SSC	Sodium chloride / Sodium citrate buffered solution
TB	Transport Buffer
VAL	Valine
V_{max}	Maximal transport velocity
v/v	Volume per unit volume
w/v	Weight per unit volume
w/w	Weight per unit weight

I. INTRODUCTION

Amino acids are the building blocks of proteins in all living things. In human and higher animals there are 10 essential amino acids that are required from the daily diet. When protein is ingested it is broken down by pancreatic proteases in the small intestine into tri- and dipeptides and also individual amino acids. These small peptides and amino acids must then be absorbed from the lumen of the small intestine into the blood. This is accomplished through a two step process involving amino acid and peptide transporters at both the brush-border membrane (BBM) and basolateral membrane (BLM).

A) TRANSPORT OR DIFFUSION

An interest in amino acid absorption originated around the turn of the century, when it was discovered that amino acids were liberated in relatively large amounts during digestion. Kutscher and Seeman [84] in 1901 observed that following the ingestion of a protein containing meal, the amino acid content of the lumen of the small intestine increased. Prior to the 1950's the prevailing view on amino acid absorption in the intestine was that it occured through simple diffusion. Evidence supporting this hypothesis came from studies by Chase and Lewis [47] demonstrating that there was no difference in the rate of absorption of the stereoisomers of several amino acids in the rat. This lack of stereoselectivity was supported by Johnston and Lewis [68] who showed that both D and L isomers of alanine disappeared at the same rate from the intestinal tract of the rabbit. More evidence supporting the diffusion model was provided by Kratzer [81] who reported that the rate of uptake of several amino acids was generally inversely proportional with molar volumes, therefore, suggesting a linear relationship existed between concentration of substrate and the rate of uptake. However, in 1937, Höber and Höber opposed the theory of simple diffusion. They declared that some special accelerating mechanism existed. Their results showed that absorption rates of amino acids were greater than could be expected from calculated rates based on the expected linear relationship [60,61]. The reason that the diffusion model persisted for a long time was primarily due to methodological problems. There was no good assay developed for distinguishing amino acids from each other. It was not until chromatographic and later radiolabelled methods were developed that these technical problems were overcome. Early tissue preparations were not suitable for studying transport characteristics. There were problems with unstirred layers and non-specific binding and the kinetic parameters were not well addressed. An assumption was made concerning the movement of amino acids from the lumen to the blood *via* a one-step process.

In 1951, Gibson and Wiseman [47], using everted sacs, found that for all the amino acids they tested the L-isomers left the rat's lumen more rapidly than the corresponding D-forms. They concluded that the amino acids were transferred to the blood by an active process which was available for the absorption of the L-amino acids. Studies conducted in humans and also found the L-isomers of amino acids were absorbed more quickly than the D-form [82]. Matthews and Smyth [186] showed that the L-isomers entered the blood faster than the D-forms *in vivo* which suggested an active process not only at the BBM, but also at the BLM. Later, Jacobs and Tarnasky [111] investigated a two stage hypothesis of trans-epithelial transport of amino acids using metabolic inhibitors. They found that these inhibitors inhibited the transport of L-lysine from the lumen into the enterocyte without simultaneous inhibition of uptake across the enterocyte and into the blood. Since transport was affected at the BBM and not the BLM they concluded that the uptake of amino acid by the epithelium and passage into the blood is mediated by two independent processes.

Following the demonstration of special mechanisms available for the uptake of L-amino acids, Wiseman [183,187] designed experiments that demonstrated the uptake of

L-isomers against their concentration gradient. This was not observed with the respective D-forms of the amino acids tested. Therefore, Wiseman concluded that there was no special system available for the uptake of the D-amino acids. Jervis and Smyth investigated the effect of concentration of L-amino acids on their rate of uptake and determined that amino acid uptake followed Michaelis-Menten kinetics [67]. The concept of transport systems for amino acids emerged. With the introduction of the everted sac technique by Wilson and Wiseman [184,185,188], competition experiments were carried out studying the action of one amino acid upon transport of another [98,99,182,189]. This led to the first classification of several distinct pathways for amino acid absorption.

A more complete picture of active amino acid transport function developed when saturation kinetics for amino acid absorption was demonstrated [67]. Early kinetic studies were conducted on various different amino acids [86,100] and each amino acid was found to follow Michaelis-Menten kinetics. Using the Lineweaver-Burk plot developed for enzyme dissociation kinetics [90], the term K_m, refers to the half-saturation value or affinity of the substrate, and V_{max}, which refers to the maximum rate of transport, were determined.

B) LYSINE TRANSPORT

A specific transport system for dibasic amino acids was first described by Haghira *et al* in the early 1960's [54]. The transport was Na^+-independent and involved the movement of arginine, lysine, and ornithine across the BBM in rat intestine. This system had a low K_m and V_{max} *in vitro* and the rates of both lysine and arginine absorption from the lumen of the rat small intestine were slow. Until recently, it was thought that lysine transport in the intestine was accomplished through a single transport system specific for dibasic amino acids designated system y^+. However, this hypothesis was challenged by Reiser and Christiansen [140], who were able to show that under certain conditions neutral amino acids could inhibit lysine uptake by the intestine. Other investigators found evidence that a neutral amino acid could stimulate the transport of dibasic amino acids in *in vitro* preparations of small intestine [143]. Using isolated cells of the small intestine, Reiser and Christiansen [142] showed that the efflux of the neutral amino acid leucine accelerated the influx of lysine on the same carrier across the BBM. This hetero-exchange system in the BBM was disproved later by Munck and Schultz [118] when they showed that intraepithelial lysine accumulation was markedly reduced by leucine . Munck [114] also found weak, non-significant inhibitory effects of dibasic amino acids on leucine transport by everted sacs. From these results he concluded that at low concentrations neutral amino acids stimulate, whereas, at high concentrations these same enhancing neutral amino acids are inhibitory. Cheeseman in 1983 [24] demonstrated that leucine exhibited *trans*-stimulation of lysine across the BLM of the anuran small intestine when present in low concentrations in the vascular bed or lumen. Stimulation in either case was suggested to occur at the outer surface of the BLM. Thus, the hetero-counter exchange phenomenon was evident at the BLM not the BBM. The BLM transporter was determined to be symmetrical as it appeared to have similar affinities for dibasic amino acids on both sides of the membrane. Both arginine and ornithine inhibited the exit of lysine into the blood when in the lumen and accelerated exit when present in the vascular bed [24]. An allosteric interaction was proposed by Lawless *et al* in 1987 [88] when *cis*-stimulation by leucine of lysine efflux in rat BLM vesicles was observed. Whatever the mechanism behind this interaction between leucine and lysine, there is most definitely an overlap in transport of both of these substrates. A simplistic view of one lysine transport system, as proposed earlier, cannot account for the numerous interactions between dibasic and neutral amino acids.

The question of Na^+-dependence was also raised by investigators. Initial studies indicated that lysine transport was entirely Na^+-independent [54]. Munck and Shultz

[117] demonstrated that the influx across the BBM appeared to be entirely Na^+-independent but the exit across the BLM exhibits a high degree of Na^+ dependence. These results were confirmed by Reiser and Christiansen in 1973 using intestinal sacs [141]. Approximately 25% of lysine transport was seen as Na^+-dependent. However, there are other investigations primarily using vesicle preparations which do not demonstrate Na^+-dependence for dibasic amino acid transport [23,92,103,105,180]. Wolfram *et al* [190] suggested that the Na^+-dependence component of dibasic amino acid transport was diet dependent. When rats were fed on a high protein diet, a Na^+-dependent component of BBM dibasic amino acid transport was observed. However, rats on a low protein diet showed no Na^+-dependence. They also demonstrated that L-leucine and L-alanine could greatly reduce the dibasic amino acid uptake into these BBM vesicles and suggested that these neutral amino acids share a common system with dibasic amino acids. In a carnivorous species such as eel, investigators [115] characterized a Na^+-dependent lysine transport system specific for dibasic amino acids and a Na^+-independent lysine transporter that accepts neutral amino acids .

Lysine transporters have been identified with characteristics different from system y^+, however, these differences have not been well addressed. Classical systems carrying dibasic amino acids have been identified in many different tissues through functional characterization (Table 1.1). The classical y^+ system appears to be ubiquitous and is characterized by Na^+-independent transport of dibasic amino acids. System y^+L was identified in the human red blood cell by Deves *et al* [39] and involves the hetero-counter exchange of Na^+-independent lysine and Na^+-dependent leucine transport. This is similar to the systems identified by Cheeseman in the BLM of anuran small intestine [24] and Reiser and Christiansen in the rat intestinal BBM [142]. Two other systems, $B^{o,+}$ and $b^{o,+}$, were identified by van Winkle et al in mouse blastocysts. The Na^+-dependent $B^{o,+}$ accepts both dibasic amino acids and a broad scope of neutral amino acids. It

Table 1.1 Classical L-lysine Transport Systems in mammalian tissues

System	Location	Characteristics of Transport
y^+	appears to be ubiquitous	Na^+-independent transporter ♦ specific for dibasic amino acids
y^+L	erthrocyte, placenta	Na^+-independent lysine and Na^+-dependent leucine transport
$B^{0,+}$	mouse blastocysts	Na^+-dependent transporter ♦ specific for dibasic amino acids and a broad scope of small and large neutral amino acids
$b^{0,+}$	mouse blastocysts	Na^+-independent transporter ♦ specific for dibasic amino acids and a broad scope of small and large neutral amino acids

appears to have a high affinity for dibasic amino acids; around $100\mu M$. The other system, $b^{o,+}$, is Na^+-independent and has similar affinities as $B^{o,+}$, but is more restrictive, not accepting larger branched chained neutral amino acids [172,173].

Cloned cDNAs have been isolated that encode proteins with lysine transport activity when expressed in oocytes of *Xenopus* laevis (Figure 1.2). The mCAT transporter family consists of mCAT 1 (system y^+), mCAT 2A, and mCAT 2B. mCAT refers to mouse cationic amino acid transporter protein. The mCAT 1 transporter, which was initially identified as the EcoR1 retrovirus receptor in mouse fibroblasts, induces Na^+-independent cationic amino acid transport, is expressed ubiquitously, and contains 12-14 transmembrane spanning domains [2,28,76,176]. All mCAT transport proteins have a high degree of sequence homology but differ in kinetic parameters. mCAT 2A has similar specificities for cationic amino acids as mCAT1, but has a K_m for lysine 10 times greater than mCAT 1. The K_m for mCAT 1 for lysine is 0.14-0.25 mM whereas the K_m for mCAT 2A has been estimated to be 2.10-5.20 mM. mCAT 2A has a higher capacity of transport when compared to mCAT 1. mCAT 2B has a K_m for lysine in the range of 0.25-0.38 mM, slightly higher than mCAT 1. mCAT 1 along with mCAT 2B resemble the " y^+ " phenotype as both systems have a high affinity for cationic amino acids and are sensitive to trans-stimulation [29,30]. rBAT (rabbit) and D2 (rat) are species variants of the same protein and exhibit Na^+-independent transport of both dibasic and neutral amino acids when expressed in *Xenopus* oocytes. Their substrate specificities are similar to $b^{o,+}$ and from Northern blots they are known to be expressed in the kidney and intestine [15,16,70,177]. However, the D2/rBAT proteins have single transmembrane spanning domains which differs in structure to earlier cloned transporters that have between 12-14 membrane spanning domains. When expressed in oocytes the D2/rbAT induced activity is similar characteristically to the endogenous activity already present in oocytes. This has led most investigators to suggest that D2/rBAT proteins

Table 1.2 Cloned cDNAs expressing lysine activities in mammalian tissues

cDNA	Location and characteristics of transport
mCAT 1	candidate y^+ transporter cloned from mouse fibroblasts - equivalent to the ecoR1 retrovirus receptor
mCAT 2A	low affinity hepatocyte isoform of mCAT1
mCAT 2B	exhibits a y^+ phenotype and equivalent to the lymphocyte TEA cell surface antigen - expressed in murine macrophage
D2	type II glycoprotein cloned from rat kidney which appears to stimulate $b^{0,+}$ activity in *Xenopus* oocytes
rBAT	rabbit kidney equivalent to D2
4F2hc	type II glycoprotein (4F2 heavy chain) cloned from human fibroblasts which appears to stimulate "y^+L" activity

function as transport regulators or activators of endogenous oocyte transporters [15,177]. D2 (rBAT) is structurally related to the human and mouse 4F2hc heavy chain cell surface antigen, and like D2, has a single transmembrane spanning region [14,136]. It induces a Na^+-independent lysine and Na^+-dependent leucine transport activity when expressed in *Xenopus* oocytes. The transport characteristics of 4F2hc induced in oocytes resembles the system y^+L in the human red blood cell. Northern blots revealed that 4F2hc is expressed in many tissues including the kidney [129].

Initially lysine transport was explained in terms of a single transport system, system y^+, in all tissues including the intestine. The recent identification of many distinct transport systems which carry lysine has led to a more complex picture of dibasic amino acid transport. Lysine transport has been shown to vary between species and between tissues within the same species. Amino acid transport appears to be fairly specific to the tissue and/or animal which could be attributed in part to the physiological role of the tissue and lifestyle of the animal.

C) GENETIC DISEASES ASSOCIATED WITH DIBASIC AMINO ACID TRANSPORT

Cystinuria

Cystinuria is an inherited transport disorder characterized by the excessive urinary excretion of cystine and the dibasic amino acids. This condition is caused by a malfunction of specific membrane transport systems located in the BBM of the proximal tubule in the kidney and the jejunum region of the small intestine. At the beginning of the nineteenth century Wollaston [192] discovered the effects of the disease naming the substance that composed an unusual bladder stone, cystic oxide. In 1908, Garrod [46] classified Cystinuria as an "in born error in metabolism" which he thought stemmed from an enzyme deficiency. It was not until the 1950's when Dent and Rose [37] hypothesized that the basis of this disease was due to a defect in the tubular absorption in the kidney of the dibasic amino acids and cystine. The intestinal defect was investigated by Milne *et al* [108] who found that cystinurics also excreted large amounts of metabolites of lysine and ornithine in the feces.

Initially, cystinuria was thought to be an autosomal recessive trait, since heterozygotes had normal excretion of both cystine and the dibasic amino acids. The first extensive genetic study by Harris *et al* in 1955 [55] found two patterns of inheritance. The first group of patients exhibited the classical autosomal recessive pattern described above. However, in the second group the heterozygotes exhibited abnormally high amino acid excretion and members this group was termed "incomplete recessives". Rosenburg *et al* [144] followed this study which investigated amino acid absorption patterns of the jejunal mucosa obtained from homozygotes. They were able to define three distinct genetic patterns. Type I corresponded to the classical type of cystinuria: transport of cystine and the dibasic amino acids is absent in homozygotes, while heterozygotes show normal excretion of these amino acids. Type II cystinuria showed an absence of dibasic amino acid transport but cystine transport was present. Type III individuals showed a reduced but not absent transport of dibasic amino acids and normal cystine uptake. The last two categories were referred to as incomplete recessive types. It was postulated that this could be evidence of more than one transport system for the dibasic amino acids and cystine in the intestinal and kidney BBM. However, other studies suggested that the three phenotypes correspond to different types of defects on the same transport system [123]. Recently, the rBAT gene, a postulated regulator of cystine and dibasic amino acid transport in *Xenopus* oocytes, has been shown to be involved in cystinuria. Mutations in this gene have been implicated as the cause for the unusual amounts of excretion of cystine in the kidney [19]. In the kidney proximal tubule there are two systems for cystine transport: a low affinity system shared with the dibasic amino acids which is present in the intestinal mucosa and a second higher affinity system not shared with dibasic amino acids and limited to the kidney [102]. Cystinuria patients that do not have dibasic aminoaciduria may have a defect in the renal high-affinity cystine carrier. Defects in the shared system would result in classical cystinuria and the Types II and III described by Rosenburg [144].

The extent of malnutrition caused by this genetic disease is source for debate. Asatoor *et al* [1] demonstrated that after an oral dose of the free amino acid lysine or a corresponding dipeptide (lysylglycine), plasma concentrations of lysine were within normal limits. However, most researchers agree that the effects of the excretion of these amino acids is regarded as more of a problem than the malabsorption. Treatments of these effects include control of urine pH, drug therapy, and for renal complications even kidney transplantations which completely corrects abnormalities in the abnormal excretion of cystine but the intestinal defect is still present [107].

Lysinuric Protein Intolerance

Another genetic disease which affects the transport of dibasic amino acids from the intestinal lumen to the blood is Lysinuric protein intolerance (LPI). In 1965 this condition was described by Perheentupa and Visakorpi [132] as the impairment of the transport of lysine, arginine, ornithine across both intestinal and renal tubular cells. Protein loading failed to increase plasma concentrations of dibasic amino acids while plasma concentrations of most other amino acids rose [22]. Oral loading of free dibasic amino acids led to only a small rise in plasma levels in patients with the defect compared to normal subjects [42,132,137]. Subsequently, it was determined from jejunal biopsy specimens that lysine efflux across the BLM to the serosa was markedly reduced leading to lysine accumulation within the cells [38]. Rajantie *et al* [38,138] demonstrated that this defect was likely due to the lack of transport of the dibasic amino acids across the basolateral membrane, showing that the Na^+-dependent transport of lysine across the

BBM was the same as in the normal subjects. Unlike cystinuria where peptide transport is unaffected, in LPI, transport of dibasic amino acids both in free and in peptide form are impaired. The dipeptide lysylglycine is transported *via* a peptide transport system into the cell across BBM and is broken down into lysine and glycine by intracellular hydrolysis [97,131]. Therefore, impaired dibasic amino acid efflux from the cell across the BLM into the blood leads to malnutrition [151].

Defective dibasic amino acid transport is also found in the proximal renal tubule [152,153]. There is an impairment of the uptake of dibasic amino acids from the glomerular filtrate into the blood due to a similar defect in basolateral dibasic amino acid transport. This results in a urinary excretion of these amino acids resulting in problems similar to those associated with cystinuria [151]. Dibasic amino acid transport in erythrocytes of LPI patients is not affected [154], which suggests that the erythrocyte dibasic amino acid transporter differs from the one found in both the BLM of intestinal and kidney epithelia. However, the genetic defect is present in skin fibroblasts [155].

LPI is inherited as an autosomal recessive trait, affecting females twice as often as males. The heterozygote trait may be undetectable [75,121] or may be expressed as a partial impairment in intestinal absorption and kidney excretion of dibasic amino acids [137]. The lack of the essential dibasic amino acids causes vomiting, diarrhea, and aversion to protein rich foods resulting in poor growth and in extreme cases, mental retardation [8]. Treatment is limited to a low protein diet supplemented with dibasic amino acids which improves growth and physical performance [22,75], but still produces diarrhea [22]. A supplement of citrulline, a precursor of several dibasic amino acids, along with lysine has proven to be the most effective treatment for correcting the abnormal amino acid pattern [75].

Investigations of both of cystinuria and LPI has helped physiologists understand more about the characteristics of lysine transport in the intestine and kidney, particularly of different, but complimentary mechanisms of transport at the BBM and BLM.

D) XENOPUS OOCYTE SYSTEM

Background

The South African species of frog, *Xenopus laevis*, is unique in that a new reproductive cycle can be initiated during any season with the administration of hormones. Oogenesis in *Xenopus* is a continuous, asynchronous process and oocytes of all stages are present in the ovary at all times in adult life [51,152]. Stage I consists of small colorless oocytes having a diameter of a mere 50-100μM and whose cytoplasm is transparent. During stage II the oocytes grow up to a diameter of 450μM and appear white and opaque. Pigment and yolk accumulation begins in Stage III and by Stage IV the animal (dark brown) and vegetal (cream colored) poles differentiate and growth occurs rapidly, yielding polarized oocytes with diameters up to 1mm. By Stage V the oocytes have nearly reached their maximum size and yolk accumulation gradually ceases. At Stage VI, oocytes are characterized by the appearance of an unpigmented equatorial band. In the last two stages, oocytes range from 1.2-1.3 mm in diameter and are ready for ovulation. The fully grown oocyte is surrounded by the following layers (starting with the innermost layer) (i) the vitelline membrane, a noncelluar fibrous layer; (ii) a layer of follicle cells connected to the oocyte plasma membrane *via* gap junctions; (iii) the theca, connective tissue layer; (iv) epithelial layer, a continuation of the ovary cell wall [40].

Both Stage V and VI oocytes are used for expression studies because of their large size and maturity. Each oocyte is capable of transcription, translation, and post-translational events. Therefore, the advantages of using *Xenopus* oocytes as an expression system is two fold: (i) their large size makes microinjection possible; (ii) their ability to translate foreign mRNA and process functional exogenous proteins.

Expressing Foreign Functional Proteins in *Xenopus* oocytes

The frog oocyte translates injected foreign mRNA with great efficiency, processing the resulting proteins, and distributing them to the correct cellular compartment. This powerful *in vitro* technique for expressing heterologous proteins was developed by Gurdon in the early 1970's [52]. The rate limiting step for mRNA translation in oocytes could be diffusion to the site of protein synthesis [52] and evidence suggests that most eukaryotic mRNAs are translated in oocytes whereas the prokaryotic mRNAs are not [130]. The reason for this is that prokaryotic mRNAs are uncapped and therefore will be degraded before translation. Such mRNAs must be artificially capped prior to injection [63]. Stability of mRNA for translation also requires a poly (A^+) tail of at least thirty residues long [122]. Injection of large amounts of mRNA does not produce corresponding large amounts of products, because of saturation of the translation machinery. Competition between different injected mRNAs as well as competition between injected and endogenous mRNAs suggest that all mRNAs use common machinery [7,87]. The oocyte appears to be able to carry out a variety of post-translational modifications, including phosphorylation, glycosylation and signal sequence removal as well as further cleavage of the resultant polypeptide [10]. Targeting of foreign proteins, especially plasma membrane proteins, has been questioned. However, in 1981, Sumikawa *et al* [157] demonstrated the presence of α-bungarotoxin binding sites, newly expressed in the oocyte surface membrane, following injection with mRNA from the electric organ of *Torpedo marmorata*. Subsequently this protein was shown to represent a functional ion channel gated by acetylcholine [10]. Therefore, the oocytes were shown to be able to properly assemble foreign multi-subunit proteins and incorporate them in a functional form into the plasma membrane.

The preparation of healthy Stage V and VI oocytes for microinjection is labour intensive, starting with the micro-dissection of these larger eggs from more immature forms. The oocytes are then subjected to collagenase treatment during which the outer ovarian epithelial layer and theca are removed. At this point the eggs separate from one another and appear fuzzy due to the follicular layer that still encompasses most of them. Treatment with a slightly hypertonic solution or mechanically with ultrafine forceps removes the follicular layer exposing the vitelline layer, which is left to provide mechanical stability. Defolliculation causes damage to oocytes as demonstrated by accompanying changes in membrane potential. Such damage can result from the rupture of membrane connections between oocytes and follicular layers, thereby producing holes in the oocyte membrane. This occurs during both manual and enzymatic defolliculation. The resting membrane potential in healthy oocytes is between -40mV and -60mV. Immediately following defolliculation, the membrane hyperpolarizes to -90mV [9,11,175]. However, after twenty four hours the resting membrane potential returns to normal. During this time period the damaged membrane is repaired leaving a healed and healthy oocyte ready for injection [35,83]. After removal of the follicular layer the oocytes are micro-injected with either mRNA into the cytoplasm [52] or cDNA into the nucleus [106]. The series of events involved in functional expression of membrane proteins in *Xenopus* oocytes is outlined in Figure 1.1. After preparation of the mRNA or cDNA *in vitro* and injection into oocytes, the genetic material undergoes translation, post-translational modification, sorting and finally insertion of a functional protein into the plasma membrane.

This functional expression system has been used to characterize many different plasma membrane proteins, the first of these being the acetylcholine receptor channel in 1981 by Sumikawa *et al* [157]. Many other channels have been expressed and characterized in oocytes including those for Na^+ [80,91], Ca^{2+} [36] , K^+ [91], and Cl^- [160]. For studying the characteristics of single ion channel currents, the patch clamp technique has been used [106]. Receptors for serotonin [3], noradrenaline [161], glycine

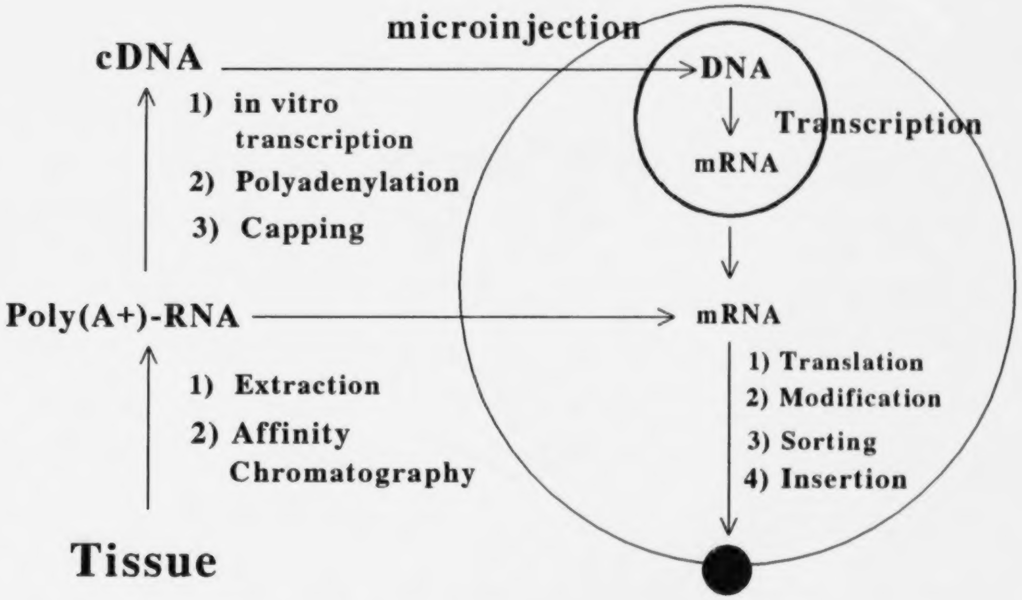
microinjection
cDNA
1) in vitro transcription
2) Polyadenylation
3) Capping
Poly(A+)-RNA
1) Extraction
2) Affinity Chromatography
Tissue
DNA
Transcription
mRNA
mRNA
1) Translation
2) Modification
3) Sorting
4) Insertion

Figure 1.1 Steps involved in functional plasma membrane protein expression in *Xenopus laevis* oocytes.

[59], glutamate [59], and olfactory sensation [34] have been expressed in oocytes. Identification of these proteins has usually involved the application of radiolabelled agonists or antagonists. The other large class of plasma membrane proteins that have been characterized by expression in *Xenopus* oocytes is nutrient and inorganic anion transporters. The Na^+-glucose co-transporter, from the rabbit intestine, was the first nutrient transport protein to be expressed by Wright's group at UCLA [58]. Equilibrative glucose transporters have also been functionally characterized in oocytes [4,48,49]. The neutral [126,165,166,168], cationic [76,104,176], and anionic [78,128,147,169] amino acid transport systems have also been expressed in oocytes as have intestinal peptide transporters [109,146]. Recently, nucleoside transporters were expressed and characterized [66,125,170] through the use of this functional expression system. The transport activity of these substrate carriers has been measured by radiotracer flux techniques [56] or by electrophysiological means [17,73,78]. The oocyte expression system can be used in the functional characterization of these plasma membrane proteins but can also be used to clone the cDNAs encoding these proteins.

Functional Expression Cloning

Isolation of cDNAs encoding receptors, channels, and membrane transport proteins classically involves purification of the protein using ligand binding or reconstitution as a functional assay, followed by N-terminal amino acid sequence analysis or the production of antibodies. Oligonucleotide probes derived from the N-terminal amino acid sequence or protein specific antibodies are then used to screen appropriate cDNA libraries. Such approaches are technically difficult for many membrane transport proteins because of their low membrane abundance, difficulty in reconstitution and lack of specific high-affinity ligands. Screening of cDNA libraries by functional expression in oocytes, or other expression systems, circumvents these difficulties and allows the cDNA isolation to proceed even if nothing is known about the molecular properties of the transporter under study. Screening of cDNA libraries by functional expression has the additional advantage that only functional (ie., potentially full length) cDNAs will be isolated. Oocytes are particularly suited for transport studies because of their large intracellular space.

Functional expression cloning in *Xenopus* oocytes was pioneered by Noma *et al* [120] and has been used subsequently to isolate cDNAs encoding many other plasma membrane proteins, including receptors [89,93], channels [45,162], and nutrient transporters [50,57,62,88,95]. A summary of the procedure is outlined in Figure 1.2. Typically, total mRNA is size-fractionated and tested for functional activity in oocytes. The mRNA fraction with the highest activity is then reversed transcribed into cDNA and a cDNA library is created in an appropriate plasmid expression vector. Positive clones are identified by progressive subdivision of the library using the oocyte system as the functional assay. A major advantage over the classical strategies is that the danger of isolating false positives, due to cross-reactivity of antibodies and nucleotide probes, can be avoided.

The Na^+-glucose co-transporter was the first solute transporter to be cloned *via* the functional expression cloning in *Xenopus* oocytes [57]. In the last five years there have been a number of other transport proteins cloned using this technique: (i) amino acid transporters [6,149,164]; (ii) a peptide transporter [44]; (iii) neurotransmitter transporters [50,124].

Other Applications

The *Xenopus* oocyte expression system can also be used to understand more about the structure-function relationships of membrane proteins. Analysis of differences between species hybrids, such the nicotinic acetylcholine receptor subtypes, enabled the identification of which subunit confers this phenotypic difference in the protein complex [158]. Also, the use of *in vitro* mutagenesis together with functional expression has

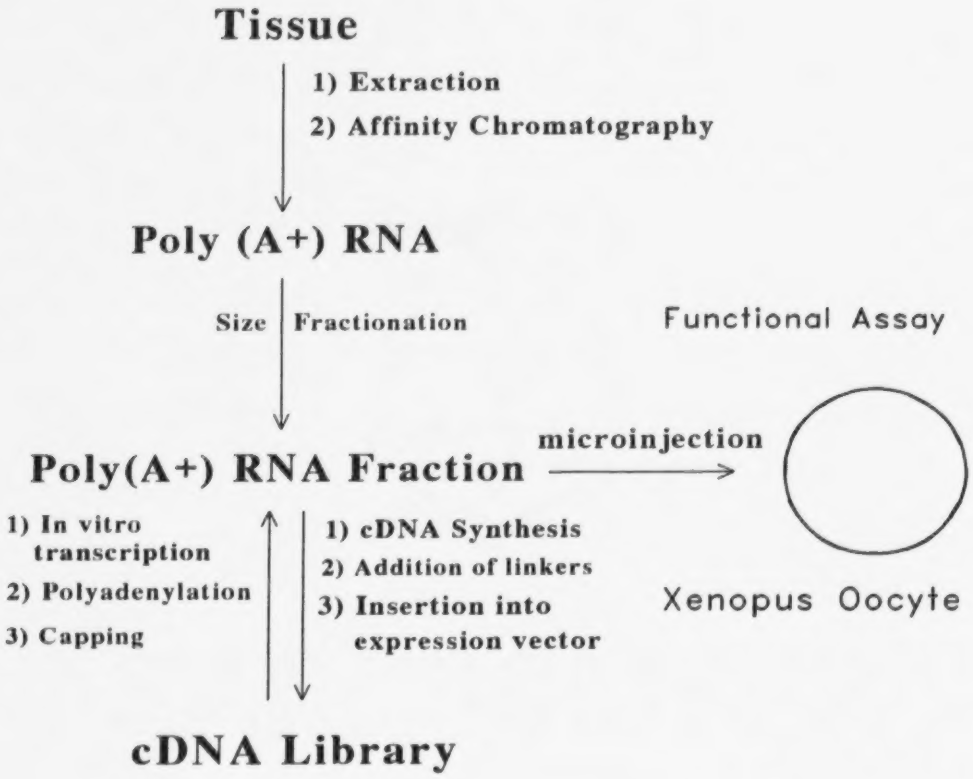
Tissue
1) Extraction
2) Affinity Chromatography
Poly (A+) RNA
Size Fractionation
Functional Assay
microinjection
Poly(A+) RNA Fraction
1) In vitro transcription
2) Polyadenylation
3) Capping
1) cDNA Synthesis
2) Addition of linkers
3) Insertion into expression vector
Xenopus Oocyte
cDNA Library

Figure 1.2 Steps involved in functional expression cloning using *Xenopus laevis* oocytes. Once the cDNA library or a pool of clones from the library tests positive in the functional assay, the clone is identified by progressive subdivision of the library or pool.

contributed to the understanding of the functional architecture of membrane proteins [64]. The oocyte expression system has been used to study all aspects of the biosynthesis of membrane proteins, including the insertion and folding in the endoplasmic reticulum [145,159]. The importance of glycosylation for assembly has also been studied and prevention of this has, in some cases, been shown to have negative effects on functional expression in oocytes [159]. Insertion of newly synthesized protein into the membrane in an incorrect orientation may explain why some proteins are not functionally expressed in the oocyte [127].

II. RESEARCH OBJECTIVE

The overall objective of the research described in this thesis was to characterize L-lysine transport systems across the rat intestine and clone the cDNA(s) responsible for these transport activities. The specific aims of this project were as follows:

1) To characterize lysine transport across the BBM of the intact rat intestine to clearly identify how many and what types of systems are involved.

2) To express the rat intestinal lysine transport systems in oocytes of *Xenopus laevis* and more thoroughly characterize the properties of these transport proteins.

3) To use the *Xenopus* oocyte system to clone the cDNA(s) that code for transport activity.

Rationale

Aim #1:

Previous studies of lysine transport across the BBM of the rat small intestine have not clearly defined the properties of the systems involved. Most of these studies used vesicle preparations which have many technical limitations causing inaccurate estimates of kinetic parameters [23]. Karasov *et al* [72] developed an intact intestinal preparation using everted segments of rat small intestine (sleeves), which was specific for studying BBM transport. This technique overcame many of the technical difficulties experienced when using vesicle preparations. Thus, the first aim was to clearly characterize and identify the lysine transport systems involved in the movement of lysine from the lumen across the BBM of the intestine, using rats as the experimental model.

Aim #2:

The use of the *Xenopus* oocyte system to express foreign proteins whether they are enzymes, receptors, or transporters is a most powerful technique. It allows one to study membrane proteins within a single cell model. Previous investigators have used this system to study the characteristics of membrane proteins which are not feasible *in vivo* or in other *in vitro* techniques [5,31,41,58,66,80,91]. Therefore, the second objective of this project was to express and further characterize those BBM and BLM lysine transport systems that have been identified in the intact rat intestine.

Aim #3:

To study individual transport systems the isolation of the individual membrane transport protein is required. Through reconstitution of the membrane protein into liposomes, membrane transport proteins can lose their transport capability. Moreover, the isolation of a single protein through this method is unlikely due to cross-reactivity of antibodies with other proteins [139,163]. Isolation of the membrane transport protein through reconstitution techniques is also limited by the absence of any high affinity ligands specific for amino acid transport. A better and more successful method is using expression cloning *via Xenopus* oocytes to clone the cDNA(s) encoding the transport proteins [15,50,57,89,96,125,167]. The strength of this method is that cDNAs which are isolated are functional (ie. potentially full length) and that no prior purification of the protein is required. Therefore, the third aim of my thesis is to use the *Xenopus* oocyte expression system to isolate the cDNA(s) from rat intestine encoding L-lysine transport activity.

III. METHODS

1) EVERTED SLEEVE EXPERIMENTS

A) TISSUE PREPARATION

These experiments used a modified version of the sleeve technique first described by Karasov *et al* in 1983 [72]. Sprague-Dawley rats (200-250 gm), fed on Wayne Rodent Blox, were anaesthetized with 0.3 ml sodium pentobarbital (65 mg/ml in propylene glycol; MTC Pharmaceuticals). The small intestine was excised and the first 35 cm of jejunum removed and flushed immediately with ice-cold PBS (137 mM NaCl, 3 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) that contained 0.1 mM phenyl-methyl-sulphonyl fluoride and then was everted (The rats were euthanised after the excision of the intestine). Each sleeve, 1.5 cm long, was mounted on a 1.5mm diameter glass rod which had grooves 5 mm and 15 mm from the end. The intestine was then tied to the glass rods at the two grooves to give an everted sleeve of intestine 1 cm long. The mounted sleeves were kept in a gassed (95% oxygen and 5% carbon dioxide mixture), ice cold Krebs solution (120 mM NaCl, 25 mM $NaHCO_3$, 4.0 mM KCl, 1.2 mM KH_2PO_4, 2.5 $MgSO_4$, 70 μM CaCl2, pH 7.4) or Na^+-free Krebs solution (with 120 mM ChCl replacing NaCl, 25 mM $ChHCO_3$ replacing $NaHCO_3$) solution until they were used (about 30 minutes maximum).

B) MEASUREMENT OF LYSINE UPTAKE

Prior to incubation, the sleeves were equilibrated in gassed Krebs solution at 37°C for 5 minutes. Incubations were performed in glass tubes (16 mm outer diameter) containing 5.5 ml of gassed medium maintained at 37°C and stirred continuously with a small bar magnet in the bottom at 900 r.p.m. The uptake was stopped after 60 seconds (unless specified differently) by immersing the rod with sleeve for 40 seconds in 100 ml of ice-cold saline stirred with a bar magnet at 900 r.p.m. The sleeves were blotted gently and then cut off the rod, weighed in a tared vial, and extracted in 0.5 ml 0.05 M $HNO_{3(aq)}$ overnight. Two 0.2 ml aliquots of extraction solution were then placed in plastic scintillation vials and 4 ml of scintillation fluid (Ready Safe, Beckman Canada Inc.) was added to each vial. ^3H or ^{14}C activity were determined by liquid scintillation counting (LS 6000 IC, Beckman Canada Inc.).

L-lysine uptake was measured by using ^3H-L-lysine (Amersham) at a concentration of 0.8 μCi/ml. Separate determinations of extracellular space were made with ^{14}C-polyethylene glycol (PEG) (Amersham) which required 5 minutes to reach equilibrium under these conditions. Measurements of extracellular space and lysine uptake were performed in duplicate (2 sleeves from the same animal per data point). In inhibition experiments when the inhibitor concentrations varied, mannitol was added to maintain a constant osmolarity.

C) STATISTICAL ANALYSIS

The lysine uptake is expressed as % uptake unless otherwise stated (i.e., nmol/g). Results for inhibition curves are presented as the average of four experiments. The error is standard error of the difference between the means (SEDM) of four experiments. Curves were fitted to data using non-linear least squares regression (Enzfitter, Elsevier-Biosoft, UK) and standard errors for estimates of the concentration required for half maximal inhibition (IC_{50}) are those derived from curve fitting. The significance of difference was tested using analysis of variance and appropriate tables of probability [21].

2) EXPRESSION STUDIES

A) MESSENGER RNA PREPARATION

(i) Total RNA Extraction:

Sprague Dawley rats (200-250 gm males), fed on Wayne Rodent Blox 8604, were anaesthetized with 0.3 ml sodium pentobarbital (65 mg/ml in propylene glycol; MTC Pharmaceuticals). The small intestine was cut at the ligament of Trietz and the first 30 cm of jejunum was removed and flushed immediately with ice-cold PBS (The rats were euthanised after the excision of the intestine). Intraluminal scrapings were frozen immediately in liquid nitrogen and ground into a fine powder before being homogenized in guanidinium thiocyanate (4M). The homogenate was centrifuged (5,000*g*, 10 minutes, 4°C) and the resulting supernatant was layered onto cesium chloride (*d* 1.7). Gradients were centrifuged (111,000*g*, 24 hr, 20°C) to yield RNA pellets. Total RNA pellets were washed twice in 80% ethanol *(v/v)*, allowed to air dry for 5 minutes, and resuspended in RNAase-free water.

(ii) mRNA Isolation:

Retrieval of Poly $(A)^+$ mRNA was achieved through by using a PolyAtract purification kit (Promega-modified protocol) or oligo-DT cellulose (Boeringher Manheim), according to the manufacturer's instructions, with the following changes.

PolyAtract mRNA Isolation Changes to the standard protocol were as follows:

Annealing of Probe

The volume of 20X SSC was doubled (changed from 60 μl to 120 μl). This was to ensure complete binding of the probe to the mRNA.

Capture and Washing of Annealed Oligo(dT)-mRNA hybrids

After addition to the washed SA-PMPs, the reaction was incubated for 30 min instead of the recommended 10 min to ensure complete binding of the hybrids to the magnetic particles.

The magnetic particles were washed in a similar fashion with 0.2X SSC rather than 0.1X SSC. This was to avoid any loss of mRNA during this last washing step before elution.

Precipitation and Resuspension

The 1 ml eluate was divided into three siliconized sterile 1.5 ml Eppendorf tubes (i.e. 333 μl per tube) and 60 μl 3M $NaCH_3COO$ (pH 5.2) and 2.5 volumes of 95% ice-cold ethanol were added to each tube. After mixing, the samples were placed in the -70°C freezer overnight or until needed. The samples were then centrifuged at 10,000g for 10 min at 4°C soon after removal from the freezer. The supernatant was carefully removed and the pellet was washed twice with 300 μl of ice-cold 80% ethanol to remove any remaining salts. The sample was centrifuged again at 10,000*g* for 5 min at 4°C and the supernatant was removed allowing the pellet to air dry for at least 5 min. The pellets were resuspended in an estimated volume of RNAse-free water to bring the mRNA concentration to 1 μg/μl. If any magnetic particles remained the tube was placed in the magnetic rack and the supernatant was removed from the SA-PMP pellet. The mRNA was stored at -70°C in 5-10 μl aliquots or was used immediately for oocyte injection.

Oligo-DT Cellulose

Solutions

2X Binding Buffer (2X BB)	20 mM Tris-HCl pH 7.5
	1 M NaCl
	2 mM EDTA
	1% SDS *(w/v)*
Binding Buffer	10 mM Tris-HCl pH 7.5
	0.5 M NaCl
	1 mM EDTA
	0.5% SDS *(w/v)*

Wash Buffer(WB)	10 mM Tris-HCl pH 7.5
	0.1 M NaCl
	1 mM EDTA
Elution Buffer(EB)	10 mM Tris-HCl pH 7.5
	2 mM EDTA

Preparation of the Oligo-d(T) Column

1. The appropriate amount of dry oligo d(T) was weighed into a sterile 10 ml Falcon tube:

   0.15 gm dry oligo d(T) for every 5.0 mg of input total RNA-1st column;
   0.05 gm dry oligo d(T) for every 5.0 mg of input total RNA-2nd column.

2. 2 ml elution buffer (EB) was added for every 0.1 g dry oligo-d(T) and the tube was slowly inverted several times to suspend the oligo-d(T).
3. The oligo-d(T) was allowed to settle briefly (1-2 min) and then the top layer of EB containing "fine" particles of oligo-d(T) was aspirated.
4. Steps 2 and 3 were repeated three more times.
5. The oligo-d(T) was then poured as a slurry in EB into the column.
6. The oligo-d(T) was washed with 5 column volumes of binding buffer.
7. The column was now ready for application of RNA.

Column Protocol

1. The RNA sample was heated to 65°C for 5 min and quickly cooled on ice.
2. An equal volume of 2X BB (37°C) was added mixed, and applied to the column.
3. The eluate was collected in a sterile tube.
4. The eluate was heated to 65°C for 5 min, quickly cooled on ice, reapplied to the column ensuring more complete binding of the mRNA to the oligo-d(T) cellulose.

5. The column was washed with 5-10 volumes of BB (37°C).
6. The column was washed with 5 volumes of WB.
7. The column was eluted with 2 column volumes of EB.
8. Steps 2-6 were repeated with the second column.
9. In this second round, the column was eluted twice with one column volume of EB.
10. The eluate was aliquoted into Eppendorf tubes and 1/10 the volume of 3M $NaCH_3COO$ and 2 volumes of ice-cold 95% ethanol were added.
11. Samples of purified mRNA were stored at -70°C overnight or until needed.
12. Precipitation and resuspension was carried out as described under the PolyAtract mRNA isolation procedure.

(iii) Qualitative Analysis:

Once isolated, the integrity of RNA and mRNA was analyzed on a denaturing 1% agarose *(w/v)* 0.66 M formaldehyde gel. A 1-2 μg aliquot was heated at 65°C for 10 minutes and cooled on ice for 5 minutes. To the sample 2μl of ethidium bromide (10mg/ml) was added and the gel was run at 80V for 1-2 hours (mini-gel) or 3-4 hours (wide-submarine gel).

(iv) Quantitative Analysis:

The concentration of RNA and mRNA was estimated by using a fluorimeter (Sequoia/Turner Model 450) [110,111]. Usually a 1-2 μl aliquot of the sample was added to 1.2 ml of an ethidium bromide buffered solution (0.5μg/ml ethidium bromide, 5 mM Tris-HCl (pH 8.1), 0.5mM EDTA,; pH 8.0). A standard of calf thymus DNA at 1 A_{260} gave a reading of 70 which correlated to 1 μg of single stranded DNA. Therefore, the amount of RNA or mRNA was determined by the following equation:

sample reading/70 x sample volume = amount of RNA or mRNA in sample

A similar procedure was used to quantify DNA. For determining the concentration of double-stranded DNA the aliquot of sample was added to an ethidium bromide buffered solution (0.5μg/ml ethidium bromide, 20 mM K_3PO_4, 0.5mM EDTA; pH 12.0). The standard of calf thymus DNA at 1 A_{260} gave a reading of 50 which correlated to 0.5 μg of DNA. The amount of DNA was determined by the following equation:

sample reading/50 x sample volume = amount of double stranded DNA

B) OOCYTE PREPARATION

Mature oocyte-positive female *Xenopus laevis* (Nasco) were anaesthetized with ice and sacrificed by a standard pithing procedure. Ovarian lobes were removed, opened and washed into modified Barth's medium (MBM: 88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 2.5 mM Na-pyruvate, 0.05 mg/ml penicillin, 0.1 mg/ml gentamycin sulphate, 10 mM HEPES, pH 7.5). Small clumps of ovarian tissue were dissected and incubated in 8 mg/ml collagenase (Type 1, Sigma Chemical Company) at 20°C for 2 hr or until oocytes became separated from the connective tissue. Individual oocytes were washed 5 times with MBM containing 0.1% *(w/v)* bovine serum albumin and then 5 times with MBM. They were then sorted and stored in MBM at 18°C and allowed to recover overnight. The next day the oocytes were incubated in phosphate buffer (100 mM K_2HPO_4, pH 6.5, 0.1% *(w/v)* bovine serum albumin) for 1 hr at 20°C, to remove remaining follicular layers. The same washing procedure was repeated as described above and the oocytes were allowed to recover for a 4-5 hr before sorting. Mature healthy stage V and VI oocytes were maintained at 18°C in MBM for twenty four hours prior to injection. Carefully controlled use of the enzyme collagenase and generous recovery times yielded oocytes that excluded trypan blue and remained visibly healthy for at least two weeks. All of the oocytes used in a particular experiment came from a single frog and each experiment was performed with oocytes from a different animal.

C) INJECTION OF OOCYTES

The oocytes were injected with either 50 nl of sample mRNA (1μg/μl) or 50 nl of RNAse-free water, unless otherwise stated. This was accomplished by using a microcapillary tip (diameter of 10-20 μM) attached to a pneumatic microinjector (Inject+Matic-System). The microcapillary tips were made by pulling microcapillary tubes on a hydraulic puller (Inject+Matic Puller) and then were broken to the proper tip diameter. The tips were then loaded with either 1.5 μl of sample mRNA or RNAse-free water. After positioning 5-10 eggs with their cream-coloured (vegetal) pole upward on a plastic petri dish, the oocytes were injected consecutively. The injected oocytes were then checked visually under the microscope, and undamaged eggs placed into sterile glass vials containing 5 ml of MBM in an incubator at 18°C. MBM was replaced daily at which time the unhealthy oocytes were removed. The oocytes that were removed had torn or punctured membranes and/or a vegetal pole that was turning dark in colour. The dead oocytes could be distinguished by their pure white appearance and were removed immediately before they could affect the healthy oocytes.

For antisense hybrid-depletion experiments [15,94,177] rat intestinal mRNA was denatured by heating at 65°C for 10 min, then hybridized to one of four synthetic oligonucleotides (DNA Synthesis Service, Department of Microbiology, University of Alberta) in 50 mM NaCl for 15 min at 42°C (1 μg mRNA and 0.25 μg oligonucleotide/μl): a) sense D2 oligonucleotide, corresponding to nucleotide positions 76-94 of rat D2 cDNA (5'CAAAGACAAGAGAGACTC-3'); b) antisense D2 oligonucleotide, complimentary to the nucleotide positions 286-301 of the rat D2 cDNA (5'GTAGCGAGCCTGGCCA-3'); c) sense 4F2hc oligonucleotide, corresponding to nucleotide positions 795 to 811 of mouse 4F2hc cDNA (5'GGTGTGGATGGTTTCCA-

3'); d) antisense 4F2hc oligonucleotide, complimentary to nucleotide positions 1146 to 1162 of mouse 4F2hc cDNA (5'GTCCCTGGCAGAGTGAA-3'). Oocytes were injected with mRNA/oligonucleotide mixtures or control mRNA as described above.

D) TRANSPORT ASSAY

After five days in MBM at 18°C, which previous work has shown maximizes expression of transport proteins [31,53,58,94,109,126] injected oocytes were assayed for amino acid transport activity using conventional radio-isotope tracer flux techniques. Initial rates of L-lysine transport (0.2 mM, 20°C) into injected oocytes were traced with L-[4,5-^{3}H]lysine (Amersham) at 7 μCi/ml using a 30 min incubation period (uptake rates were constant during this interval). Flux assays were performed in transport buffer (TB: 100 mM NaCl or 100 mM ChCl; 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5). For assays performed in the absence of sodium, oocytes were first washed and then incubated for one-half hour in ChCl TB prior to uptake experiments. At the end of the flux incubation, the extracellular label was removed by five rapid 1 ml ice-cold washes in ChCl TB, removing the supernatant between washes. All washes were completed within three minutes. Individual oocytes were dissolved in 5% *(w/v)* sodium dodecyl sulphate (SDS), allowing the subsequent assay of intracellular L-lysine by liquid scintillation counting (LS 6000 IC, Beckman Canada Inc.).

E) SUCROSE DENSITY GRADIENT CENTRIFUGATION

Total rat intestinal mRNA (150 μg) was denatured by heating to 65°C for 10 min and then cooling immediately at 4°C on ice for about 15 min. It was then loaded onto a 10 ml 5-25% (*w/v*) sucrose density gradient made according to a protocol outlined by Baxter-Gabbard [12]. The mRNA was at a concentration of $\approx$ 3 μg/μl (in about 50 μl). After centrifugation at 97,000g for 20 hr at 4°C, 0.25 ml fractions were collected by gravity using a fraction collector system into siliconized 1.5 ml Epppendorf tubes. Consectutive fractions were pooled, giving twenty 0.5 ml fractions. Then, 60 μl of 3M $NaCH_3COO$ (pH 5.2) and 900 μl of ice-cold ethanol (95%) were added and the tubes were mixed well. The samples were allowed to precipitate overnight at -70°C and were then centrifuged at 10,000*g* for 30 minutes at 4°C. The supernatants were removed and the mRNA pellets were washed twice in 300 μl of 80% ice-cold ethanol (*v*/*v*) and were allowed to air dry for 5 min at room temperature. The mRNA was resuspended in 10 μl of RNAse-free water and stored at -70°C.

F) GEL ELECTROPHORESIS

Samples of the mRNA fractions collected from the sucrose density gradient were loaded onto a 1% (*w*/*v*) agarose-formaldehyde (0.66 M) gel, and run with ethidium bromide at 40 V for 11.5 hours. The sizes of the mRNAs in different fractions were determined by laser densitometry (Gelscan XL, Pharmacia) using poly $(A)^+$-tailed RNA molecular weight markers (GIBCO/BRL) as standards.

G) STATISTICAL ANALYSIS

Results are presented as means of flux determinations from ten individual oocytes ± the standard error of the mean (SEM). Where differences of means are presented, the error is the standard error of the difference between the means (SEDM). The significance of differences was tested using either Student's *t* test or analysis of variance and appropriate tables of probability [21]. Curves were fitted to data using non-linear least squares regression (Enzfitter, Elsevier-Biosoft, UK), and standard errors for estimates of K_i, or for estimates of the concentration required for half maximal inhibition (IC_{50}), are those derived from curve fitting.

3) CLONING STUDIES

A) CONSTRUCTION OF cDNA LIBRARY

A mRNA size fraction (median 2.3 Kb) was reversed transcribed using the Riboclone (Promega) cDNA synthesis system with an *Xba* primer-adaptor consisting of oligo(dT) adjacent to an *Xba1* restriction site. Digestion of the resulting double-stranded cDNA with *Xba1* gave orientation-specific DNA with a 5' *EcoR1* terminus and a 3' *Xba1* terminus. cDNAs ≥ 2 Kb were ligated in the *EcoR1* and *Xba1* restriction enzyme sites of the plasmid expression vector pGEM-3Z (Promega) (Figure 2.1) and transformed into *E.coli* (JM 109) to give a cDNA library containing 6,800 primary recombinants. This cDNA library was constructed by Dr. Qi Quan Huang for the purpose of nucleoside transport expression cloning studies [194].

B) SUBCLONING STRATEGY

The cDNA library was divided into 22 pools of 700-800 clones and plated onto agar plates (containing 200 μg/ml Ampicillin). Replicates were made by overlaying each plate with Whatman filter paper. Both sets were allowed to grow at 37°C overnight. Total cDNA from each pool was tested for lysine transport activity by injection of corresponding cRNA into oocytes (see SEC.*3*C,*3*D,*3*E). Individual colonies from the master plates of transport-positive pools were individually seeded into the wells of 96-well flat bottom microtitre plates (with 100 μl of LB medium containing 200 μg/μl ampicillin) to produce a grid system. A replicate of this grid system was stored at -70°C (100 μl of glycerol was added prior to freezing and, when needed, wells were scraped still frozen; these microtitre plates were not allowed to thaw). The first round of screening of the positive pools was carried out by testing the rows and columns of the corresponding microtitre plates *via* a grid system. In order to minimize the number of

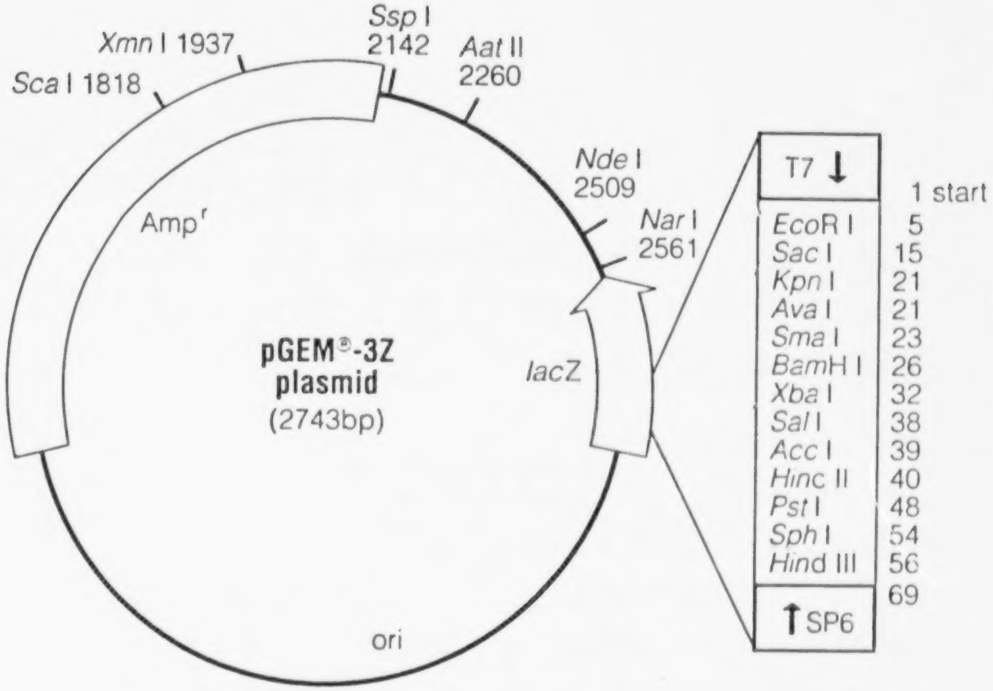
Ssp I
2142
Xmn I 1937
Aat II
2260
Sca I 1818
Nde I
2509
Nar I
2561
Amp^r
pGEM®-3Z
plasmid
(2743bp)
lacZ
ori
T7
1 start
EcoR I 5
Sac I 15
Kpn I 21
Ava I 21
Sma I 23
BamH I 26
Xba I 32
Sal I 38
Acc I 39
Hinc II 40
Pst I 48
Sph I 54
Hind III 56
69
SP6

Figure 2.1 Restriction map of the plasmid vector pGEM-3Z. It is 2.7 kb in size and is bidirectional with both T7 and SP6 promoter regions. cDNA inserts were linked at the *EcoR1* and *XbaI* restriction sites in the *lacZ* region of the plasmid.

rows tested during the first screening, each row corresponded to 4 rows on the microtitre plate. For pool #47 there were 12 columns and 27 rows tested for lysine transport activity by expression in *Xenopus* oocytes. Plasmid cDNA was isolated from each row and column (SEC.*3*C,*3*D) and cRNA was made through *in vitro* transcription (SEC.*3*E). Once a positive row(s) and column(s) was (were) identified, the remaining group of clones were subdivided further in order to isolate a single positive clone.

C) ISOLATION OF PLASMID DNA

1) Initial pools

The plasmid DNA from the initial 22 pools was isolated by growing up each pool in liquid culture for 12-15 hr in 5 ml of LB medium (containing 200 μg/ml of Ampicillin). The samples were centrifuged at 4000*g* for 15 min. The supernatant were removed and the pellets were resuspended in 1 ml of sterile M.Q. water and transferred to an Eppendorf tube. The pellet was then resuspended in 0.3 ml of the P1 buffer from the Qiagen Mega-Script Kit. The protocol from this kit was followed and the resulting plasmid DNA was resuspended in 20 μl of sterile M.Q. water. The plasmid DNA was quantitated using fluorimetry (SEC.*2*A).

2) Microtitre Plates

Plasmid DNA from each row and column was isolated by taking a 10 μl aliquot from the corresponding wells of each column or row and growing up the bacteria on agar plates (containing 200 μg/ml Ampicillin) at 37°C for 12-15 hours. The resultant bacteria were removed from the plate and resuspended into sterile M.Q. water and the pellet was washed twice with 1 ml sterile water before resuspending into 0.3 ml of the P1 buffer from the Quiagen Mega-Script Kit. The protocol from this kit was followed and the resulting plasmid DNA was resuspended in 20 μl of sterile M.Q. water. The plasmid DNA was quantitated using fluorimetry (SEC.*2*A). Plasmid DNA was isolated from the frozen replicate plates by scraping the frozen cells and growing them on an agar plate (containing 200μg/ml Ampicillin) at 37°C for 12-15 hours. The rest of the protocol was the same as described above.

3) Individual clones

Individual clones from the wells of the microtitre plates were streaked on a agar plate (containing 200 μg/ml Ampicillin) and grown up 12-15 hours at 37°C. Several individual colonies were chosen and each was restreaked onto agar plates, allowed to grow overnight, and plasmid DNA was isolated by the same method outlined in SEC. C1 above.

D) LINEARIZING THE PLASMID DNA

The isolated plasmid DNA was digested with the restriction enzyme *Xba1* to linearize the DNA for *in vitro* transcription. A 5 μg sample of plasmid DNA was added to 10 μl of 10X transcription buffer and 2 μl of *Xba1* restriction enzyme (20 units/μl) and the volume was adjusted to 100 μl using sterile M.Q. water. After mixing thoroughly, the mixture was allowed to incubate at 37°C for 2 hr. After incubation, an equal volume of saturated phenol/Tris-HCl buffer (pH 8.0) was added to the sample and it was mixed vigorously. Following centrifugation at 10,000*g* for 5 min the aqueous layer was removed and the phenol layer was washed once with an equal volume of sterile M.Q. water. The aqueous layers were pooled and an equal volume of chloroform/isoamyl alcohol (24:1) was added to remove the remaining traces of phenol. After mixing and centrifugation at 10,000*g* for 5 min, the aqueous layer was removed and the linearized plasmid DNA was precipitated by the addition of 1/10 volume of 3 M $NaCH_3COO$ (pH 5.2) and 3.5 volumes of ice-cold 95% ethanol.

E) *IN VITRO* TRANSCRIPTION

The linearized plasmid DNA (1-2 μg) was *in vitro* transcribed using the MegaScript Ambion system with addition of 3μl of CAP analog (8 units/μl, Ambion) ensured that the newly transcribed cRNA was capped with a poly (A^+) tail and therefore resistant to oocyte degradation. The cRNA was then precipitated by addition of LiCl, according to the Ambion Megascript protocol, and the resultant cRNA pellet was resuspended in RNAse-free water and subjected to a phenol/chloroform extraction (see SEC.*3*D). The cRNA was precipitated as described in SEC.*3*D and the pellet was resuspended in RNAse-free water and analyzed quantitatively as in SEC.*2*A. The cRNA was stored in 8-10 μl aliquots (1μg/μl) at -70°C prior to injection.

F) INJECTION OF cRNA AND TRANSPORT ASSAY

The injection of cRNA into oocytes follows the same procedure outlined in SEC.*2*C. Transport assays were the same as described in SEC.*2*D.

G) PCR AMPLIFICATION

Single stranded cDNA was reversed transcribed from rat jejunal poly $(A)^+$ RNA using oligo $(dT)_{12\text{-}18}$ as a primer. The primers used for PCR amplification of D2 and 4F2hc were the same two pairs of sense and antisense oligonucleotides synthesized for the antisense hybrid depletion experiments described in SEC.*B*3. Reaction mixtures (100 μl) contained 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% *(w/v)* gelatin, 25 ng cDNA, 100 pmol of each primer and 2.5 units of *Taq* polymerase (Perkin-Elmer Inc.). Amplification was accomplished by incubation at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute. After 30 cycles, 15 μl of each reaction mixture was separated on a 2% *(w/v)* non-denaturing agarose gel containing 0.5 μg/ml of ethidium bromide. The amplification products were subcloned into pCR™II vector

(Invitrogen). Positive clones were isolated and sequenced by using the *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems Inc.) as described in SEC.3I.

H) DIAGNOSTIC PCR

In parallel with screening the PGEM-3Z cDNA library for transport activity by expression in *Xenopus* oocytes, the pools were also tested for D2 and 4F2hc activity by diagnostic PCR (Minicycler, MJ Research). The cDNA from each pool, row/column, or isolated cDNA was subjected to the same amplification procedure described in the last previous section. Results from a representative experiment PCR reaction using cDNA prepared from rat jejunal mRNA are shown in Figure 2.2 (panel 2), demonstrating amplification of a 0.2 Kb PCR product with D2 primers and a 0.3 Kb PCR product with the 4F2hc primers. The deduced amino acid sequences of these PCR products and their alignments with mouse 4F2hc is shown in Figure 2.2 (panel 1).

I) SEQUENCING AND HYDROPATHY ANALYSIS OF RAT INTESTINAL 4F2hc

The testing of the rows and columns for lysine transport activity identified a single positive colony (well 8A) from which a plasmid with an insert of 1.8-Kb insert was isolated. Diagnostic PCR gave a reaction product with 4F2hc but not D2 primers. The insert was sequenced in both directions by overlapping deletions generated by exonuclease III (Erase-a-base System, Promega) and verified by sequencing with synthetic oligonucleotides. Sequencing by the dideoxynucleotide chain termination method was performed by *Taq* DyeDeoxy terminator cycle sequencing with an automated Model 373A DNA Sequencer (Applied Biosystems, INC., DNA Sequencing Lab, Department of Biochemistry, University of Alberta). Hydropathy analysis of the deduced amino acid sequence was performed by the method of Kyte and Doolittle [85]. Sequencing and hydropathy analysis of 4F2hc were conducted in co-operation with Sylvia

1)

```
(A)

                          241
mouse 4F2                 FRDVGKLMNAPLYLAEWQNITKNLSEDRLLIAGTESPDLQQIVNILESTS
                           : :::: :: :::::::::::: :::::::::: :::::::::::::::
4F2-PCR product           VRHVGKLANASLYLAEWQNITKNFSEDRLLIAGTASPDLQQIVNILESTS
                          1

                          291
                          DLLLTSSYLSNSTFTGERTESL VTRFLNATGSQWCSWSVSQAGADTSFI
                          ::::::::::   :::   : : :   :::::: ::::::::::  ::::
                          DLLLTSSYLSQPAFTGEHAE LLVIKYLNATGSRWCSWSVSQAGLLTSFI
                          51

                          340
                          PDH LLRLYQLLLFTLPG
                          :   : ::::::::::::
                          PAQFL RLYQLLLFTLPG
                          100


(B)

                          11
rat D2                    IQMSMKGCRTNNGFVQNEDIQEQDPDSRDTPQSNAVSIPAPEEPQLKVVR
                          ::::::::::::::::::::::::::::::::::::::::::::::::::
D2-PCR product            IQMSMKGCRTNNGFVQNEDIQEQDPDSRDTPQSNAVSIPAPEEPQLKVVR
                          1

                          61
                          PYAGMPKEVLFQFSGQARY
                          :::::::::::::::::::
                          PYAGMPKEVLFQFSGQARY
                          51
```

2)

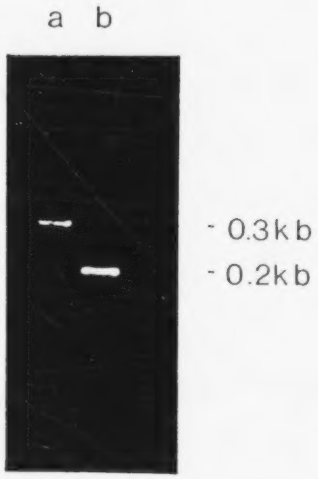

Figure 2.2 **1)** Deduced amino acid sequences of the 4F2hc and D2 PCR products from the rat jejunum. (A) Alignment of the amino acid sequences (one letter code) of mouse fibroblast 4F2hc top and the rat jejunal 4F2hc-PCR product(bottom) (B) Alignment of amino acid sequences of rat kidney D2(top) and the rat jejunal D2-PCR product (bottom). Gaps are indicated by spaces. **2)** An agarose gel (2% (w/v)) of the 4F2hc and D2 PCR products from total rat jejunal cDNA. Lanes (a) and (b) contain the PCR products amplified from rat jejunal cDNA with 4F2hc primers and D2 primers, respectively.

K) EFFLUX EXPERIMENTS

Oocytes were injected with either 50 ng of rat intestinal 4F2hc cRNA or 50 nl of water (See SEC.2C) and left for five days to incubate in MBM at 18°C. On the fifth day the oocytes were preloaded for 3 hr with L-lysine by placing them into 1 ml of NaCl TB containing 0.2 mM of unlabelled L-lysine and L-[4,5-^{3}H]lysine at 28 μCi/ml. After the loading step the oocytes were washed by 5 rapid 1 ml washes in ice-cold ChCl TB and left on ice until the start of efflux. Twenty five oocytes were used for each condition and the efflux medium was either 1.5 ml of NaCl or ChCl TB at room temperature. After each time interval two 5 μl aliquots was taken from each well and placed into a scintillation *via*l with 4 ml of scintillation fluid, and counted for extracellular L-[4,5-^{3}H]lysine (LS 6000 IC, Beckman Canada Inc.). After 60 min, when a baseline was observed, 75 μl of 100 mM unlabelled L-leucine was added to give a final concentration of 5 mM. During efflux, the oocytes were continually mixed at 150 r.p.m. on a Gyratory Shaker (Model G2, New Brunswick Scientific) to prevent unstirred layers and ensure uniform mixing.

IV. RESULTS

1) CHARACTERIZATION OF LYSINE TRANSPORT USING JEJUNAL SLEEVES

A) DETERMINATION OF THE KINETIC PARAMETERS

The everted sleeve technique was preferred over other whole tissue or vesicle methods because of its many advantages [72]. This technique was designed to specifically look at transport across the BBM. The extracellular space corresponding to diffusion between enterocytes was corrected for by subtracting the uptake of PEG, which is known not to be taken into the cells. The uptake of lysine (0.2 mM) by rat jejunal sleeves was linear with respect to time over 120 seconds both in the presence and the absence of Na^+ as shown in Figure 3.1a,b. The incubation time used to determine initial rates of transport in the subsequent experiments was set at 60 seconds. Figure 3.1 also indicates a reduction in lysine uptake when Na^+ was removed from the extracellular media. This result suggested that there are two components of lysine transport in the BBM; a Na^+-dependent (29%) and a Na^+-independent component (71%). To determine the apparent K_m for lysine transport in the presence and absence of Na^+, the uptake of lysine was (60 second flux) was determined over a range of concentrations. The results in Figure 3.2 indicate a high affinity saturable component of uptake both in the presence and absence of Na^+, with apparent K_m values of 0.37 ± 0.06 mM and 0.39 ± 0.07 mM respectively. The V_{max} values calculated from curve fitting were 193 ± 10.1 nmol/g in NaCl and 151 ± 9.15 nmol/g in ChCl media, and the difference reflects the presence of a Na^+-dependent component (Shown in Figure 3.2). This saturable component of uptake corresponds to a carrier-mediated process rather than simple diffusion. With the apparent K_m values in the 0.30-0.40 mM range, a working concentration of 0.20 mM was

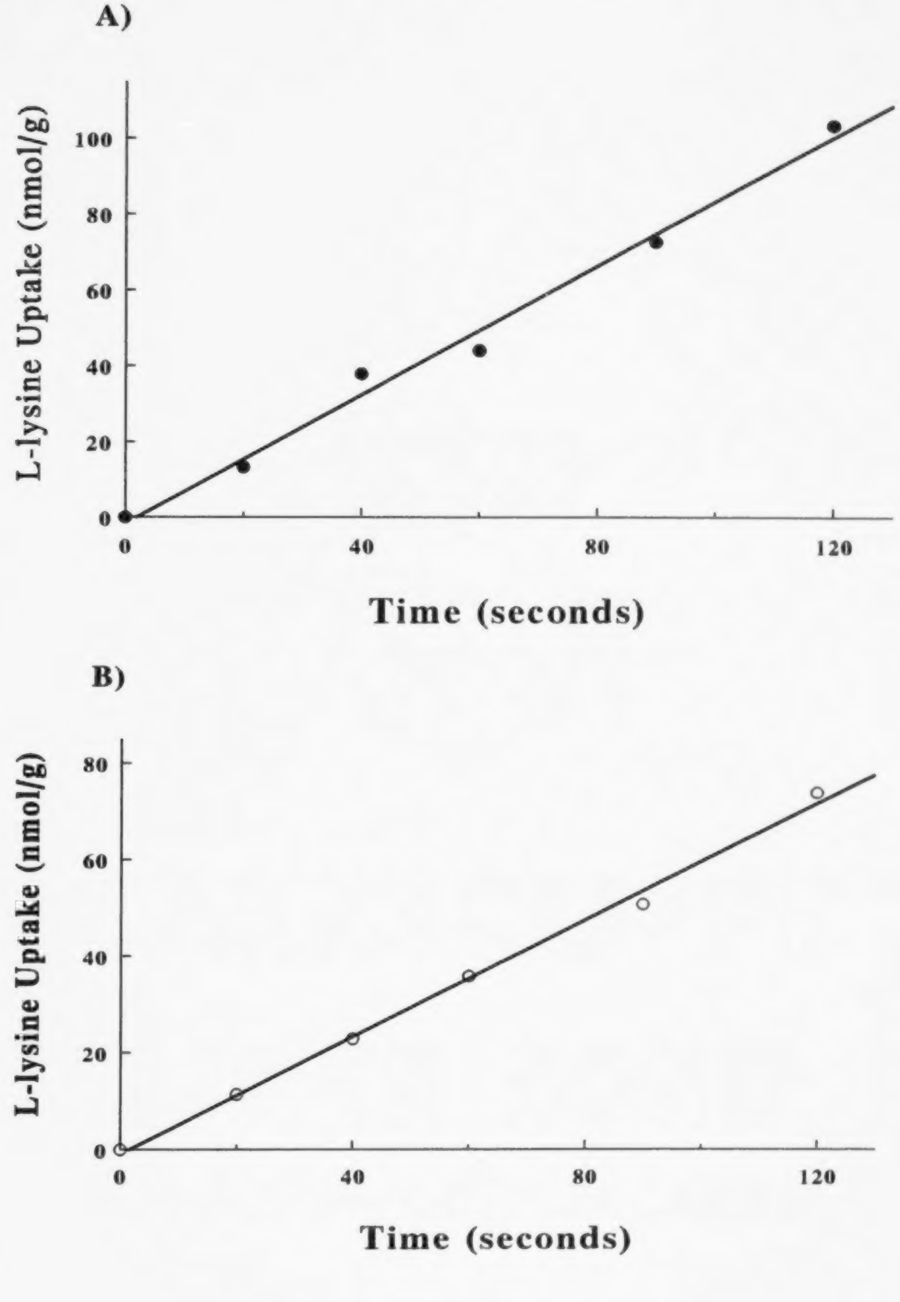

Figure 3.1 The net uptake of L-lysine into intestinal sleeves measured as a function of time. Data shown are taken from one representative experiment. The uptake was performed in the either (A) NaCl or (B) ChCl medium. The linearity was determined by linear regression analysis using computer program Enzfitter (Elsevier).

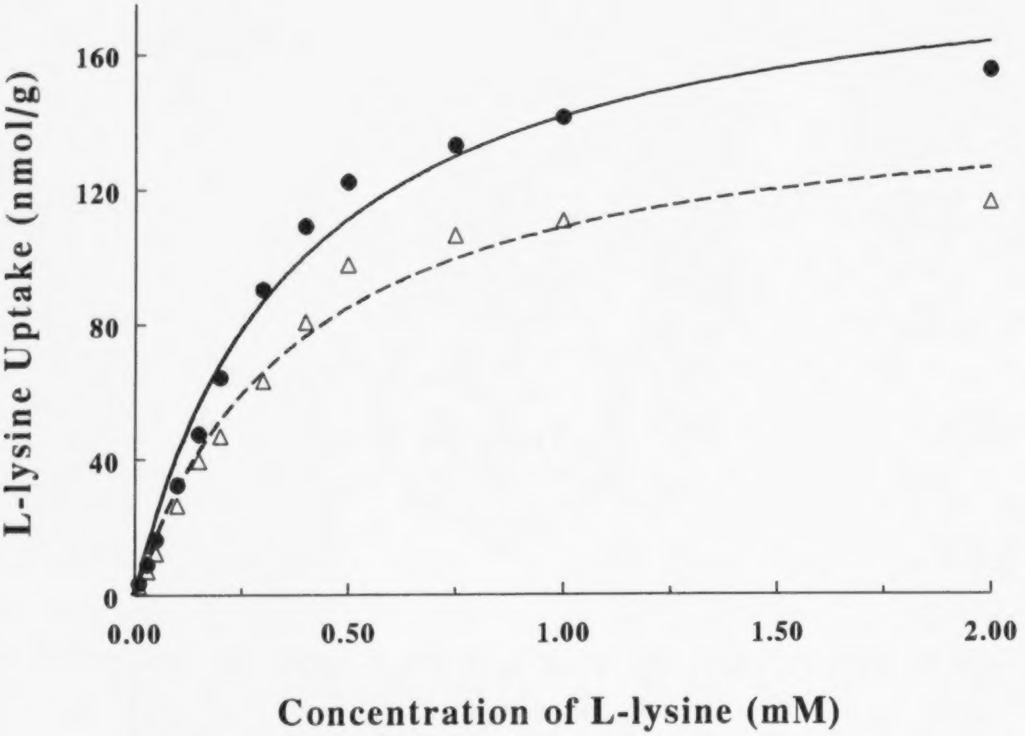

Figure 3.2 The net uptake of L-lysine into intestinal sleeves measured over increasing concentrations of L-lysine. Data shown are taken from one representative experiment. The uptake (60 seconds) was done in the presence of NaCl (closed circles) or ChCl (open triangles) medium. Curves were fitted by non-linear regression analysis.

used in all proceeding experiments. The physiological concentration of amino acids within the lumen has been determined to be within the range of 1-2 mM [25].

B) COMPETITION EXPERIMENTS WITH OTHER AMINO ACIDS

In order to determine the substrate selectivity of the BBM transporters, competition experiments using non-radioactive L-amino acids were employed. Since the absolute uptake varied for each animal, the uptake was expressed as % of control for the inhibition experiments. Almost complete inhibition of L-lysine uptake (0.2 mM) was observed with 5 mM L-arginine both in the presence and in the absence of Na^+ indicating again that transporter activity, rather than diffusion, is responsible for net lysine uptake by rat jejunal sleeves (Figure 3.3). The concentration of L-arginine required for fifty *per cent* inhibition (IC_{50}) of the uptake was 0.35 ± 0.06 mM in NaCl and 0.37 ± 0.08 mM in ChCl media. This supports the suggestion of high affinity systems for dibasic amino acid transport in the BBM of the small intestine. Some neutral amino acids were tested as inhibitors of lysine uptake to determine whether BBM lysine transporters were specific for dibasic amino acids. L-leucine, which, like lysine is a C4 amino acid, was also able to inhibit lysine uptake both in the presence and absence of Na^+, however, a portion of the uptake was insensitive to L-leucine inhibition (Figure 3.4). This leucine insensitive component of lysine uptake (33%) could be system y^+, which has a specificity for only dibasic amino acids. L-leucine inhibited the other (major) component of lysine uptake IC_{50} values of 0.36 ± 0.08 mM in NaCl and 0.29 ± 0.05 mM in ChCl media. Since there was significant inhibition of the lysine uptake with the neutral amino acid leucine, I investigated whether other neutral amino acids could also have an effect. Neutral amino acids specific for system L (a Na^+-independent transporter of mostly larger neutral amino acids like phenylalanine), system $B^{o,+}$ (valine), and system $b^{o,+}$(alanine) were tested. L-alanine exhibited moderately high affinity inhibition both in NaCl and ChCl

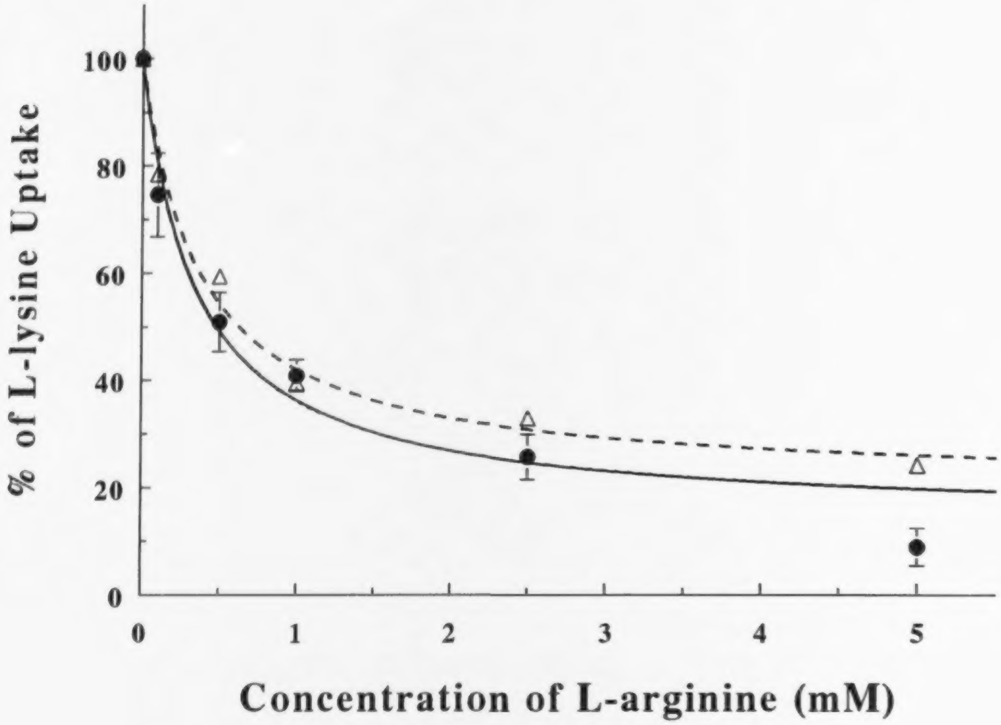

Figure 3.3 L-Arginine inhibition of L-lysine uptake into intestinal sleeves. Data shown are the means of four individual experiments. The uptake was performed in NaCl (closed circles) and ChCl media (open triangles). Curves were fitted by non-linear least squares regression. Error bars are the SE of difference between the means.

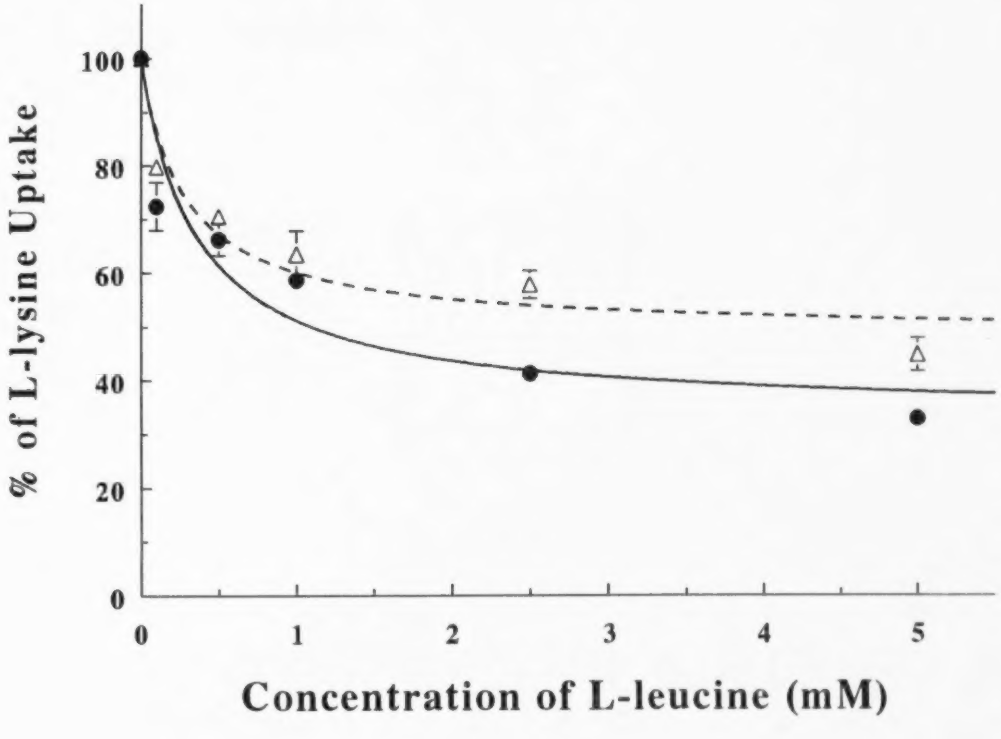

Figure 3.4 L-leucine inhibition of L-lysine uptake into intestinal sleeves. Data shown are the means of four individual experiments. The uptake was performed in NaCl (closed circles) and ChCl media (open triangles). Curves were fitted by non-linear least squares regression. Error bars are the SE of the difference between the means.

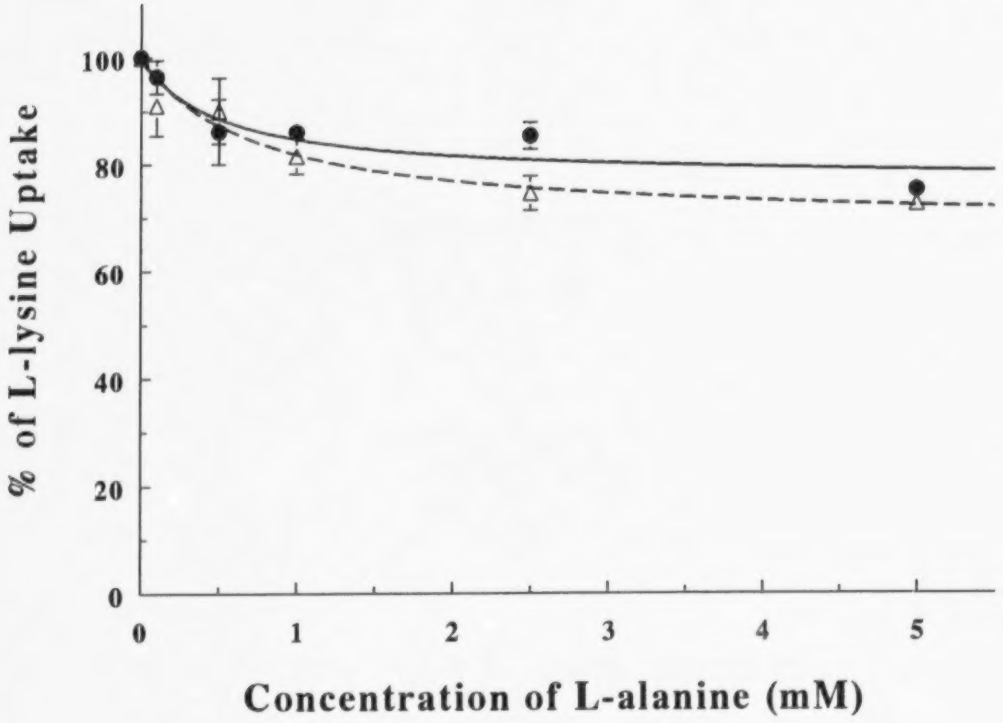

Figure 3.5 L-alanine inhibition of L-lysine uptake into intestinal sleeves. Data shown are the means of four individual experiments. The uptake was performed in NaCl (closed circles) and ChCl media (open triangles). Curves were fitted by non-linear least squares regression. Error bars are the SE of the difference between the means.

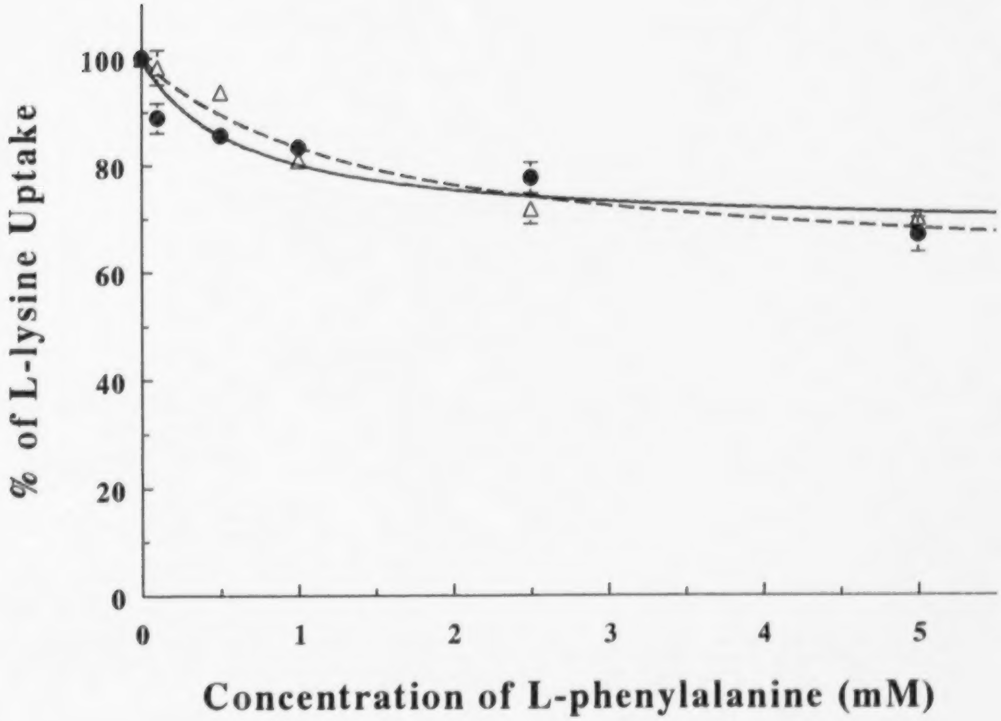

Figure 3.6 L-phenylalanine inhibition of L-lysine uptake into intestinal sleeves. Data shown are the means of four individual experiments. The uptake was performed in NaCl (closed circles) and ChCl media (open triangles). Curves were fitted by non-linear least squares regression. Error bars are the SE of the difference between the means.

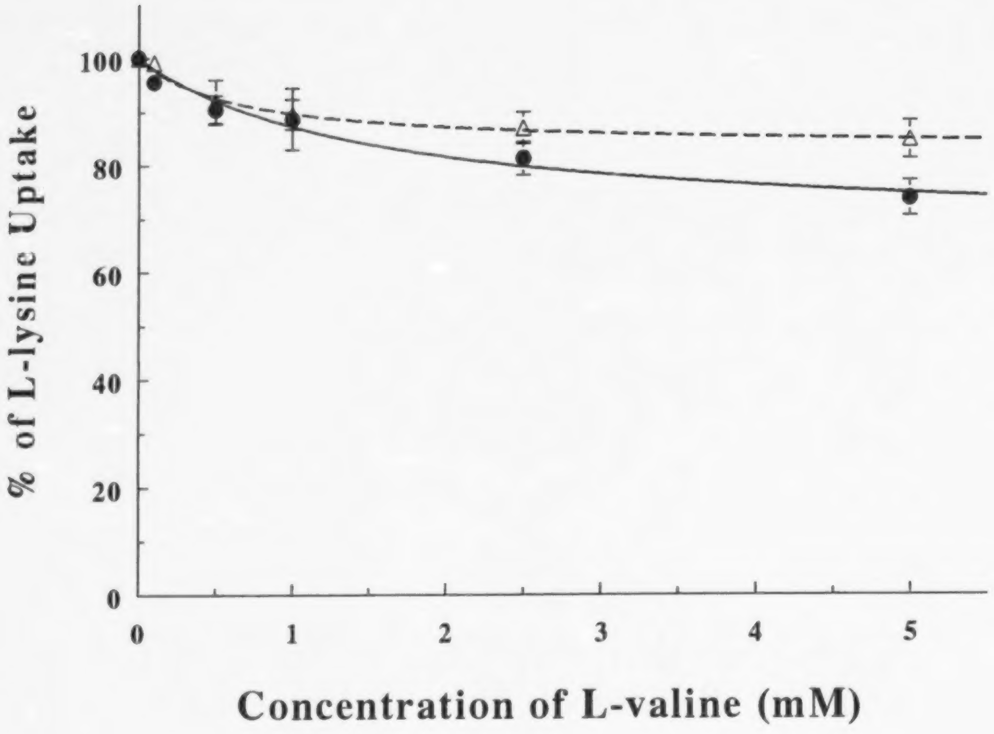

Figure 3.7 L-valine inhibition of L-lysine uptake into intestinal sleeves. Data shown are the means of four individual experiments. The uptake was performed in NaCl (closed circles) and ChCl media (open triangles). Curves were fitted by non-linear least squares regression. Error bars are the SE of the difference between the means.

media with IC_{50} values of 0.52 ± 0.15 mM and 0.76 ± 0.32 mM respectively. However, it was only able to inhibit a small portion of the L-lysine uptake; 23 ± 4% in NaCl and 32 ± 5 % in ChCl (Figure 3.5). The IC_{50} values for L-phenylalanine inhibition were 0.64 ± 0.24 mM in the presence of Na^+ and 1.47 ± 0.58 mM in the absence of Na^+. This amino acid at 5 mM was able to inhibit L-lysine uptake by 32 ± 7% in the presence of Na^+ and 41 ± 6% in the absence of Na^+ (Figure 3.6). A similar pattern was also seen with L-valine which produced a low affinity inhibition of lysine uptake in both NaCl and ChCl media (Figure 3.7). The L-valine concentrations for half maximal inhibition of lysine influx were 1.62 ± 0.51 mM in the presence of Na^+ and 0.67 ± 0.13 mM in the absence of Na^+. Addition of 5 mM L-valine inhibited the L-lysine uptake by 33 ± 4% in NaCl and 17 ± 2% in ChCl media. Therefore, only L-arginine and L-leucine exhibited substantial, high affinity inhibition of lysine uptake into the intestinal sleeves.

C) ADDITIVE INHIBITION EXPERIMENTS

In the above competition experiments L-leucine appeared not to completely inhibit the carrier mediated lysine uptake. However, at a concentration of 20 mM the remaining lysine flux was abolished by L-leucine, suggesting that L-leucine inhibited a second component of lysine uptake, but with a much lower affinity. The following experiments investigated whether or not other neutral amino acids could also reduce this component of lysine uptake, which was marginally inhibited by 10 mM leucine. If a reduction in the remaining lysine uptake was observed this would suggest that other neutral amino acids also interact with lysine transport with a low affinity. In figure 3.8A further inhibition was obtained with L-alanine and L-valine but not L-phenylalanine, in the presence of Na^+, which could suggest the involvement of a $B^{o,+}$ like system in the BBM of the rat intestine. Inhibition by these three neutral amino acids was not evident in the absence

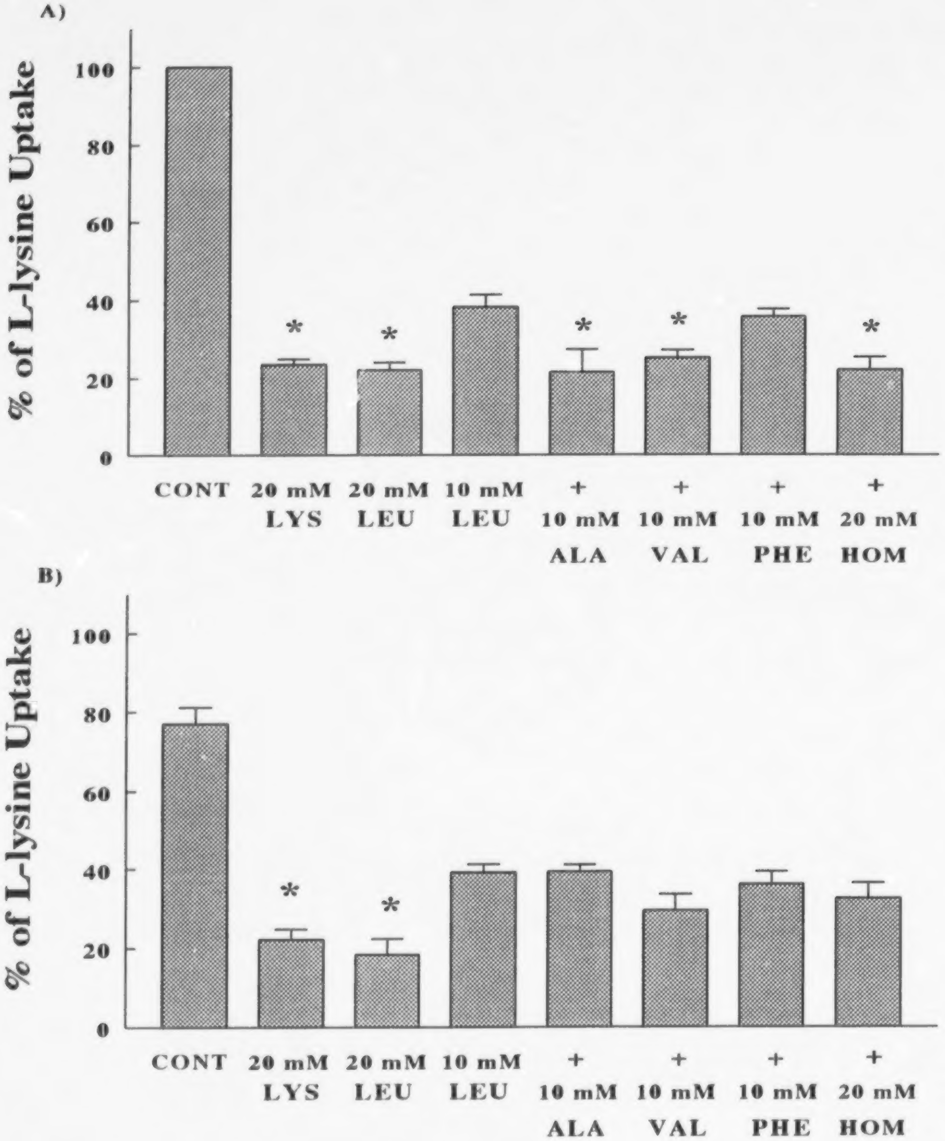

Figure 3.8 Additive inhibition of the net uptake of L-lysine into intestinal sleeves. Data shown are from the average of four individual experiments. The uptake was performed in either NaCl (A) or ChCl (B) medium. The last 4 bars measure L-lysine uptake in the presence of 10 mM L-leucine + the amino acid indicated. The uptake in ChCl is expressed as a % of the uptake in NaCl. Error bars are the SE of the difference between the means. Significant differences, relative to inhibition by 10 mM L-leucine, were determined by analysis of variance and are indicated by * ($p=0.05$) .

of Na^+ (Figure 3.8B). Also as shown in Figure 3.8A homoserine, in the presence of Na^+, significantly inhibited this component although not in the absence of Na^+. This result suggested the involvement of system y^+, which is known to be inhibited by L-leucine and by homoserine only in the presence of Na^+ [181].

The results from all of these competition experiments suggested that there are up to four different high affinity transport systems for L-lysine in the rat intestinal BBM. These could include: 1) a Na^+-dependent system which resembles $B^{0,+}$; 2) a Na^+-independent system which has a high affinity for L-leucine even in the absence of Na^+; 3) a Na^+-independent system which has a low affinity for L-leucine in the presence or absence of Na^+ and a low affinity for L-alanine and L-valine in the presence of Na^+; and possibly 4) a system which has high affinity for alanine, valine, and phenylalanine in the presence or absence of Na^+ (see Figures 3.5, 3.6, & 3.7).

Given the apparent complexity of carrier mediated lysine uptake across the rat jejunal BBM it is not going to be possible to definitively discriminate all of the pathways involved using competition experiments. Therefore, another approach was needed and in the following section the use of the oocyte expression is described.

2) EXPRESSION STUDIES

The *Xenopus* oocyte expression system was used to overcome the many problems associated with intact tissue and vesicle preparations. This system would potentially enable the characterization and identification of both the BBM and BLM lysine transport systems. More importantly, functional expression in *Xenopus* oocytes may be an effective way to clone cDNA (s) that encode these intestinal transport proteins [50,57,79,125,134].

A) DETERMINATION OF KINETIC PARAMETERS

A time course shown in Figure 3.9 determined the net lysine uptake in mRNA-injected oocytes to be linear for up to 60 minutes, validating the use of a 30 minute incubation. The apparent K_m of lysine transport was determined in both the presence and absence of Na^+ by measuring lysine uptake over a range of concentrations. In Figure 3.10, after the injection of total rat intestinal poly $(A)^+$ mRNA, the net lysine uptake followed a saturable carrier-mediated curve with an apparent K_m of 0.17 ± 0.02 mM in NaCl and 0.22 ± 0.06 mM in ChCl media. The K_m was calculated to be $\approx$ 0.20 mM for both components which is the same as the lysine concentration used in the sleeve and subsequent expression experiments.

The amount of intestinal mRNA injected into each egg to obtain maximal expression of lysine transport activity was determined by injecting varying amounts of intestinal mRNA into oocytes. Figure 3.11 shows that injection of 25 - 50 ng mRNA/egg resulted in maximal net expression both in the presence and absence of Na^+. Therefore, to ensure maximal expression, in all subsequent oocytes experiments 50 ng/egg of mRNA or cRNA was used (ie. standard amount). Another determining factor in oocyte expression is the length of time after injection and before assaying for transport activity. Most previous work has shown that maximal expression for transporter proteins is obtained 5-7 days after injection [31,53,58,94,109,126] . Using the standard amount of intestinal mRNA injected per oocyte, transport activity was assayed over a 9-day period (Figure 3.12). Significant expression was observed on Day 1, however, maximal expression was obtained after the seventh day and remained at this level past the ninth day. The uptake of lysine by water injected controls remained constant throughout the nine day experiment (Figure 5.1). A substantial amount of expression was obtained after five days, in both the absence and presence of Na^+, the incubation period was chosen as the standard for all other experiments.

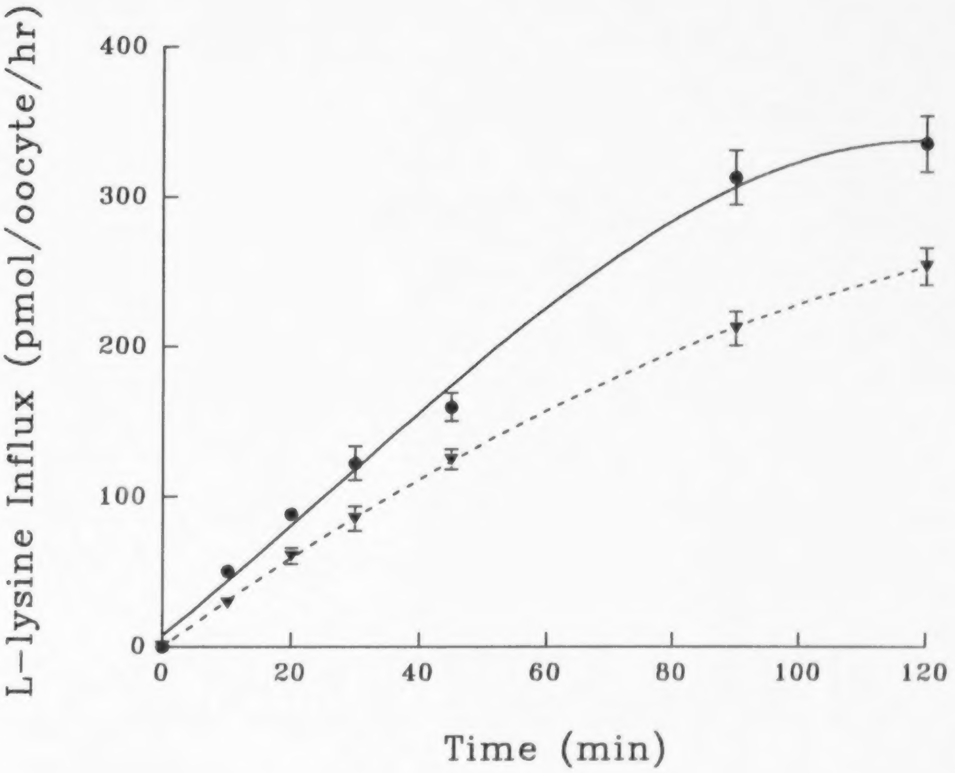

Figure 3.9 Influx of L-lysine (0.2 mM) into isolated oocytes of *Xenopus laevis* as a function of time. Results shown are for net flux due to injection of intestinal mRNA. Fluxes were performed in NaCl TB (closed circles) and ChCl TB (closed triangles). Each data point is difference between means of uptake into 10 individual oocytes injected with intestinal mRNA and 10 individual oocytes injected with water. Errors bars are the SE of the mean. All oocytes came from one frog.

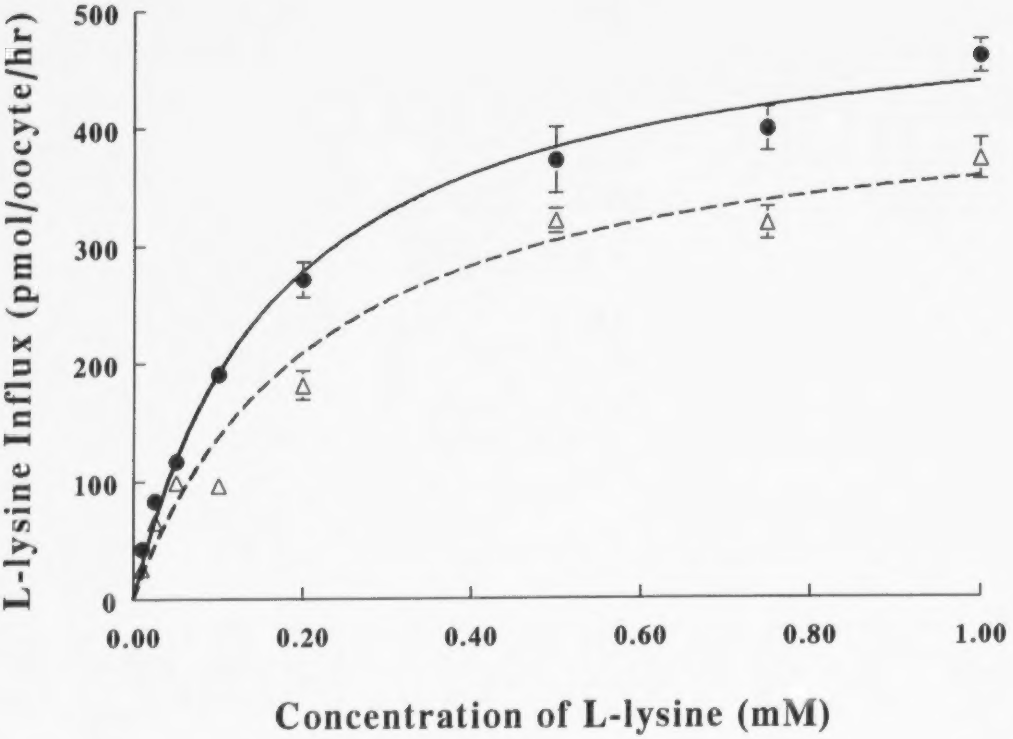

Figure 3.10 Influx of L-lysine into isolated oocytes of *Xenopus laevis* as a function of L-lysine concentration. Results shown are for net flux due to injection of intestinal mRNA. Fluxes were performed in NaCl TB (closed circles) and ChCl TB (open triangles). Each data point is difference between means of uptake into 10 individual oocytes injected with intestinal mRNA and 10 individual oocytes injected with water. Curves were fitted by non-linear regression analysis. Errors bars are the SE of the mean. All oocytes came from one frog.

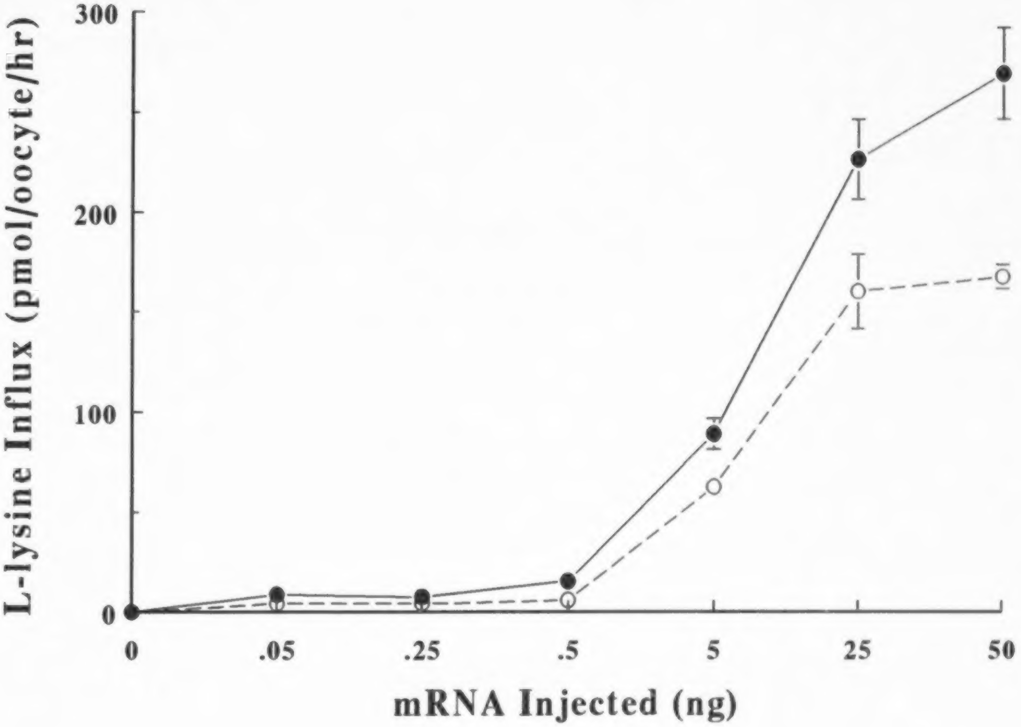

Figure 3.11 Influx of L-lysine (0.2 mM) into isolated oocytes of *Xenopus laevis* as a function of the amount of mRNA injected. Results shown are for net flux due to injection of different amounts of intestinal mRNA. Fluxes were performed in NaCl TB (closed circles) and ChCl TB (open circles). Each data point is the difference between the means of uptake into ten individual oocytes injected with varying amounts of mRNA and ten oocytes injected with water. Error bars are the SE of the difference between the means. All oocytes came from one frog.

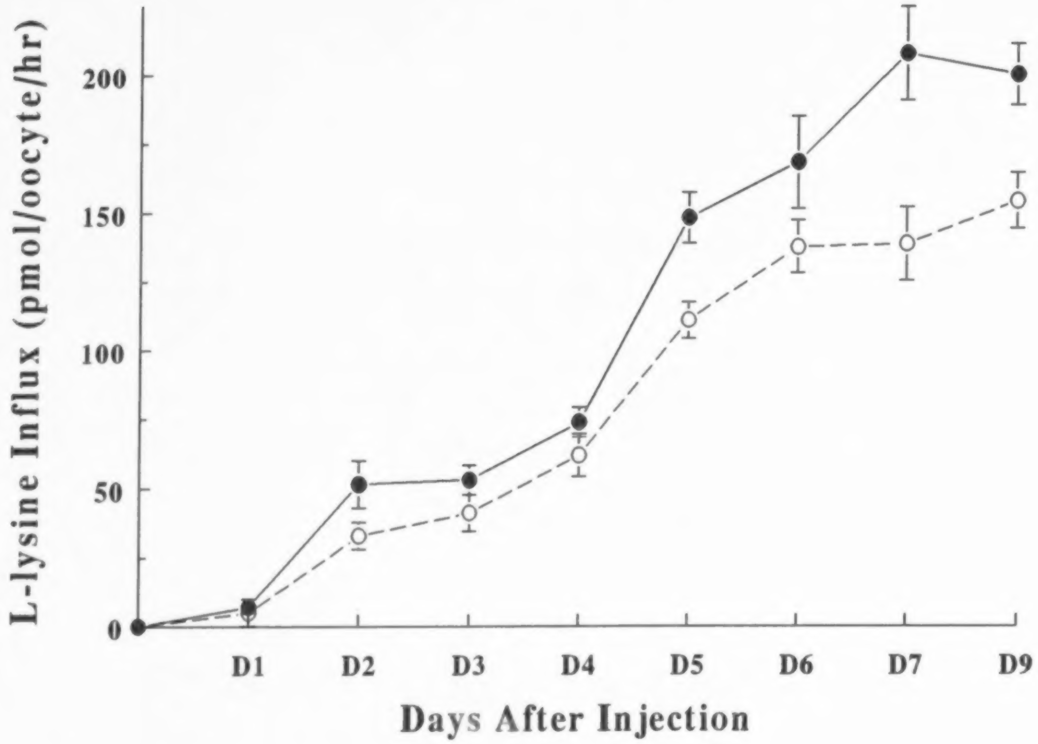

Figure 3.12 Influx of L-lysine (0.2 mM) into isolate oocytes of *Xenopus laevis* as a function of post-injection period. Results shown are for net flux after 2-9 days following injection of intestinal mRNA. Fluxes were performed in NaCl TB (closed circles) and ChCl TB (open circles). Each data point is the difference between the means of uptake into ten individual oocytes injected with mRNA and ten oocytes injected with water. Error bars are the SE of the difference between the means. All oocytes came from one frog.

B) EXPRESSION OF L-LYSINE TRANSPORTERS IN OOCYTES

The injection of rat intestinal poly(A)$^+$ into oocytes resulted in a 3- to 4.5 fold higher influx of L-lysine (0.2 mM) than was measured in control oocytes injected with water. A representative experiment is shown in Figure 3.13. Total lysine influx was 382 ± 23 pmol/oocyte^{-1}/hr^{-1} compared with 85 ± 3 pmol/oocyte^{-1}/hr^{-1} for oocytes injected with water. In the experiment shown, net expression due to the injection of intestinal mRNA was 297 ± 3 pmol/oocyte^{-1}/hr^{-1}. With four different preparations of mRNA and oocytes from four different frogs, expressed exogenous L-lysine influx ranged from 150 ± 7 to 297 ± 23 pmol/oocyte^{-1}/hr^{-1}. The Na$^+$ dependence of this expressed flux was investigated by replacing Na$^+$ with choline in the transport buffer, as described in METHODS SEC.2D. In the absence of Na$^+$ the net expressed flux shown in Figure 3.13 was reduced by 38%, revealing both Na$^+$-dependent and Na$^+$-independent components of induced lysine transport. When fluxes were performed in the presence of excess (10 mM) unlabelled L-arginine, the net expressed lysine influx was reduced by 94%, leaving a small residual expressed flux of 19 ± 4 pmol/oocyte^{-1}/hr^{-1}. Therefore, both the Na$^+$-dependent (38%) and Na$^+$-independent (56%) components of expressed lysine transport could be inhibited by excess arginine, implying that transporter activity accounted for the induced flux. The physical effects of microinjection were not responsible for increased lysine influx because controls were injected with the same volume of water; uptake in those oocytes was not significantly different from uptake in uninjected oocytes (Figure 5.2). In 8 separate experiments, Na$^+$-dependent lysine uptake accounted for 38 ± 5% of the total net expressed flux. In Figure 3.13b, endogenous lysine transport into oocytes injected with water was reduced by 9% in the absence of Na$^+$. Excess (10 mM) arginine inhibited only 27% of the total endogenous flux in NaCl TB, implying that endogenous transport is largely *via* a low affinity route which might include a substantial diffusive component. In 8 separate experiments, removal of Na$^+$

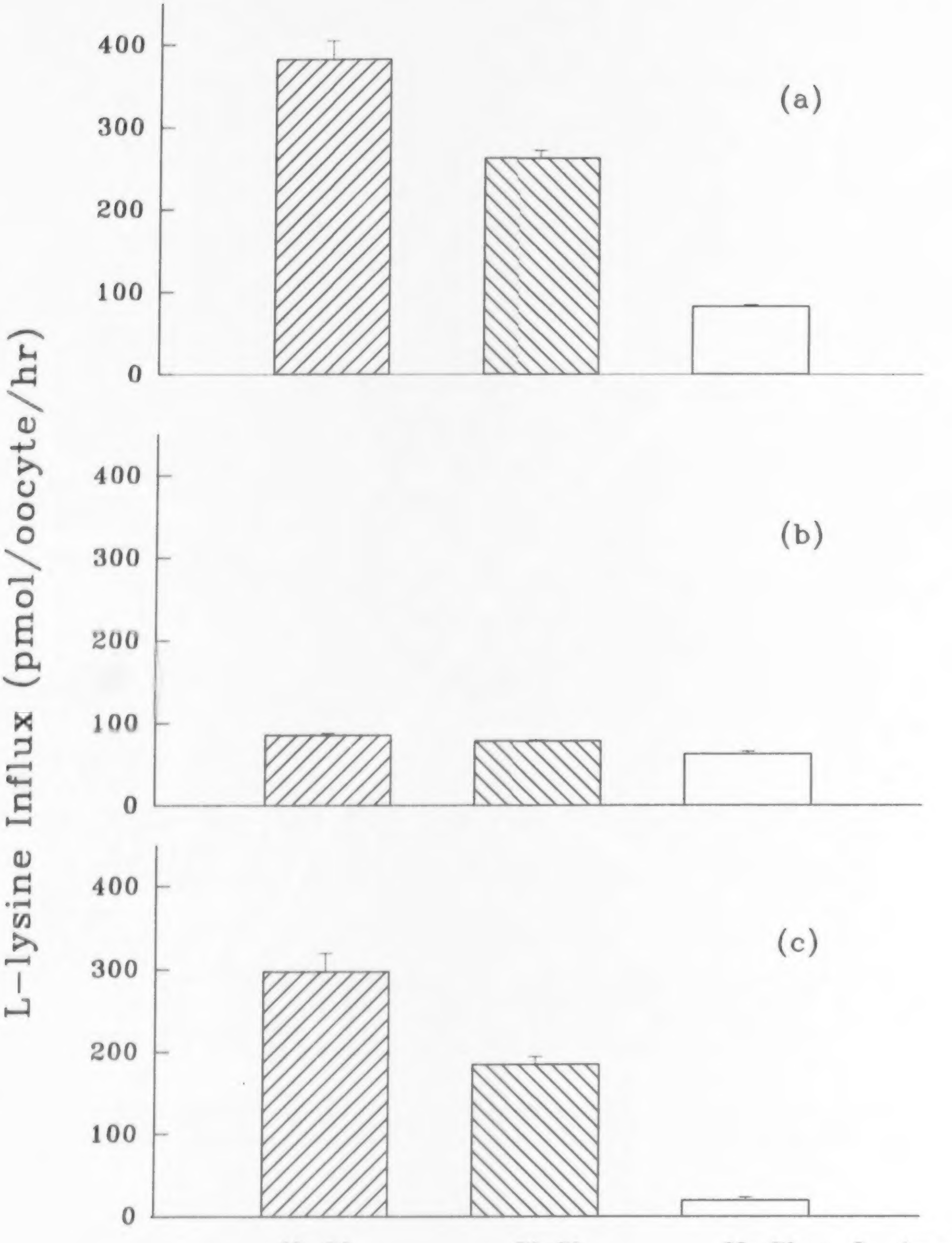

Figure 3.13 Influx of L-lysine (0.2 mM) into isolated oocytes of *Xenopus laevis*. (a) Injected with rat intestinal mRNA; (b) injected with water; (c) net expression calculated by (a) - (b). Uptake was measured in NaCl TB, choline chloride TB or NaCl TB with 10 mM L-arginine. Results are means ± SEM, n = 10 for each condition. All oocytes came from one frog.

caused a mean reduction in endogenous lysine uptake of 24 ± 8%, while 10 mM arginine inhibited influx by 27 ± 2%. To determine whether the method of follicle removal had any effect on the level of endogenous lysine transport detected, oocytes were denuded manually, without the use of collagenase treatment. These oocytes showed endogenous lysine uptake of 106 ± 4 pmol/oocyte/hr, which was within the range found for oocytes from different frogs treated with collagenase in our study (57 ± 2 to 136 ± 5 pmol/oocyte/hr, $\underline{n}=17$).

C) COMPETITION EXPERIMENTS WITH OTHER AMINO ACIDS

The characteristics of exogenous expressed lysine influx were investigated using unlabelled amino acids which might interact with intestinal lysine transport systems. The influx of 0.2 mM lysine was measured in the absence or in the presence of various concentrations of unlabelled L-arginine or L-leucine, both in oocytes injected with mRNA and those injected with water. The resulting inhibitory characteristics of L-arginine on the net expressed lysine flux are shown, for one typical preparation, in Figure 3.14. L-arginine was a potent inhibitor of exogenous lysine influx into oocytes, in the presence of NaCl, capable of abolishing the expressed lysine uptake in a concentration-dependent manner, confirming that transporter activity, rather than diffusion, was responsible for net uptake (Figure 3.14a). The concentration of L-arginine required for fifty *per cent* inhibition of the expressed systems was 0.16 ± 0.04 mM. Therefore, arginine was clearly inhibiting both Na^+-dependent and Na^+-independent routes of lysine influx with high affinity. The ability of arginine to inhibit the net expressed flux was not changed when choline replaced sodium in the transport buffer (Figure 3.14b). Also, the pattern of inhibition did not change; the concentration required for 50% inhibition was 0.19 ± 0.05 mM in choline chloride TB and arginine was able to inhibit almost all of the induced flux. L-leucine was also able to inhibit net lysine influx in NaCl TB (Figure 3.15a), although the concentration required for half maximal inhibition was 0.31 ± 0.02 mM, double that for arginine (0.14 ± 0.01 mM for this batch of oocytes, 95% inhibition at 5mM arginine). In the absence of sodium, the inhibitory ability of leucine was substantially reduced; it could only inhibit 67% of the net Na^+-independent lysine influx, and did so with reduced affinity, the concentration required for half maximal inhibition being 0.83 ± 0.07 mM (Figure 3.15b). Since the neutral amino acid leucine appeared to interact with lysine transport in a Na^+-dependent fashion and with high affinity, we investigated whether L-alanine could do so too. A representative experiment is shown in Figure 3.16. In the absence of sodium, a portion (25 ± 2%) of the net expressed lysine transport was inhibited by alanine with an apparent K_i of 0.09 ± 0.02 mM. Maximum inhibition occurred at relatively low alanine concentrations leaving the remaining Na^+-independent lysine transport unaffected by alanine (Figure 3.16b). In the presence of sodium, the Na^+-dependent component of lysine transport was also assayed, and this expression could be inhibited by alanine to a greater extent (43% ± 3%), (Figure 3.16a). It is not clear whether the additional inhibition by alanine in NaCl was of a Na^+-dependent or Na^+-independent component of lysine transport. However, unlike the situation with respect to leucine inhibition of lysine influx, the magnitudes of the alanine-insensitive components of lysine influx were comparable in the two media. This suggested that alanine interacts mainly with the Na^+-dependent component of lysine transport. The high affinity interaction of leucine with lysine transport in the presence of sodium led us to test proline, L-phenylalanine and L-cysteine as potential inhibitors of lysine transport. At 1 mM these unlabelled amino acids did not inhibit net expressed lysine influx significantly in either NaCl or choline chloride TB (Figure 3.17). The apparent ability of proline to stimulate lysine influx in choline chloride TB was not statistically significant at the 1% level, but was just significant at the 5% level ($t = 3.13_{df, 18}$). Other amino acids were tested at the 1 mM concentration in both the presence

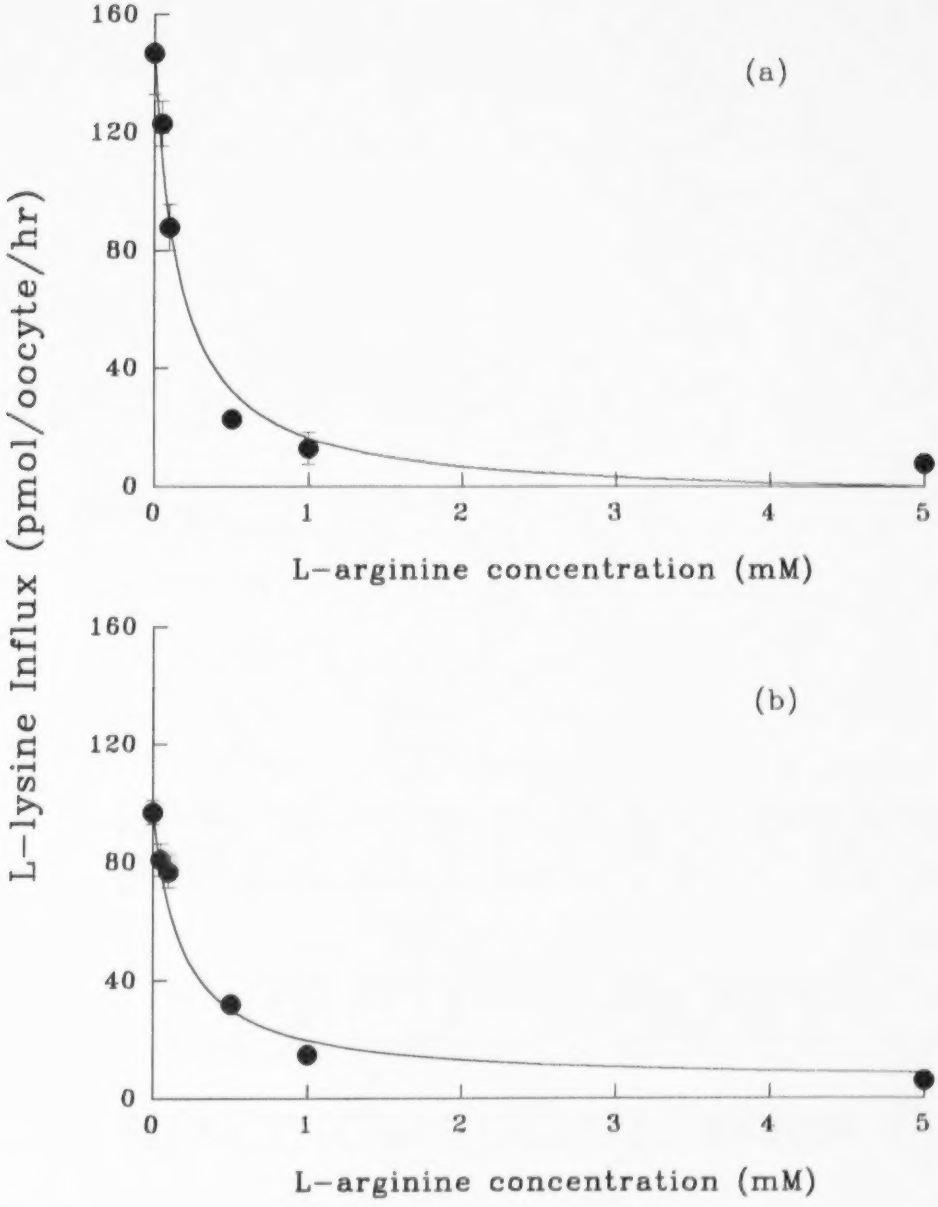

Figure 3.14 L-arginine inhibition of L-lysine (0.2 mM) influx into isolated oocytes of *Xenopus laevis*. Results shown are for net flux due to injection of intestinal mRNA. Fluxes were performed in (a) NaCl TB; (b) ChCl TB. Each data point is the difference between the means of uptake into ten individual oocytes injected with mRNA and ten oocytes injected with water. Curves were fitted by non-linear least squares regression. Error bars are the SE of the difference between the means. All oocytes came from one frog.

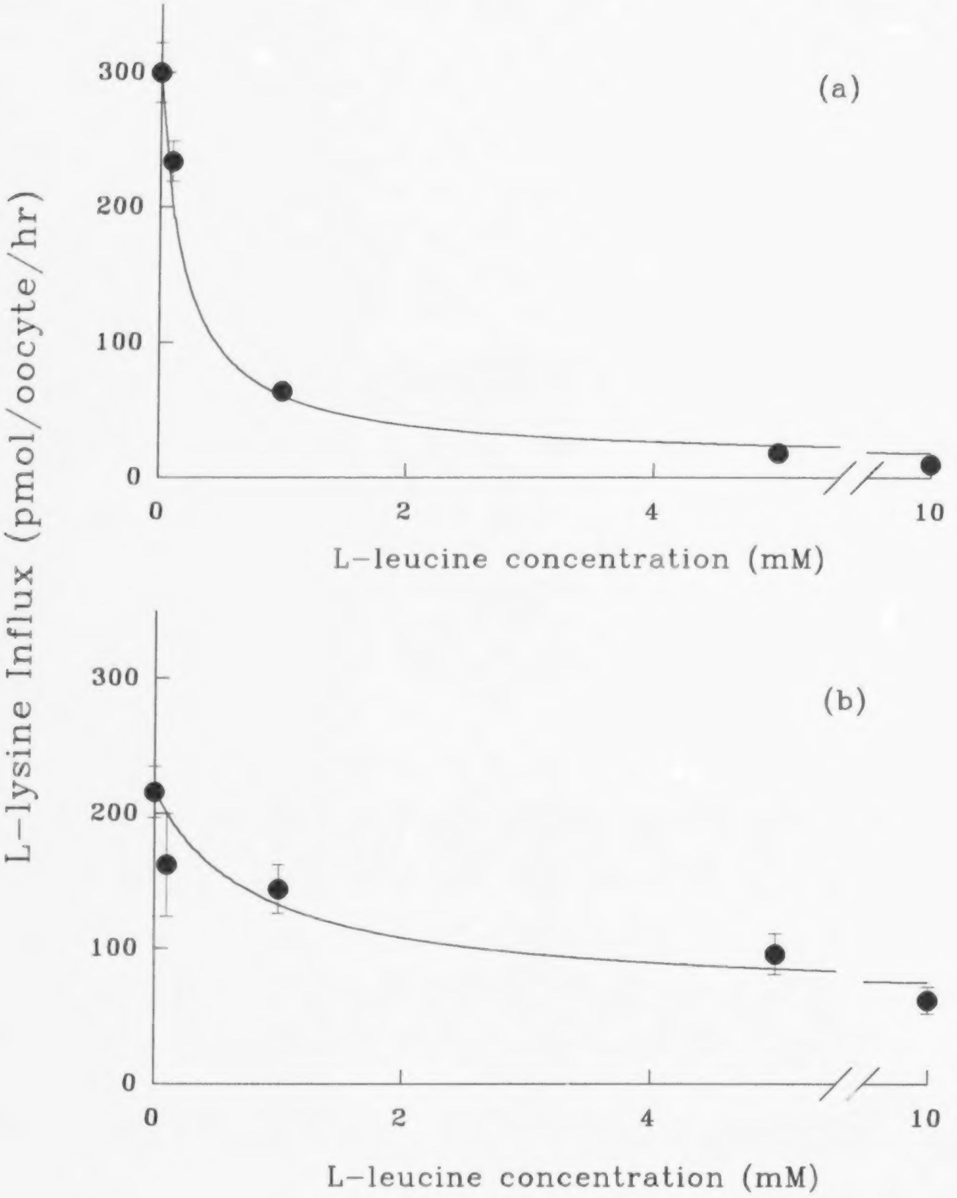

Figure 3.15 L-leucine inhibition of L-lysine (0.2 mM) influx into isolated oocytes of *Xenopus laevis*. Results shown are for net flux due to injection of intestinal mRNA. Fluxes were performed in (a) NaCl TB; (b) ChCl TB. Each data point is the difference between the means of uptake in ten individual oocytes injected with mRNA and ten oocytes injected with water. Curves were fitted by non-linear least squares regression. Error bars are the SE of the difference between the means. All oocytes came from one frog.

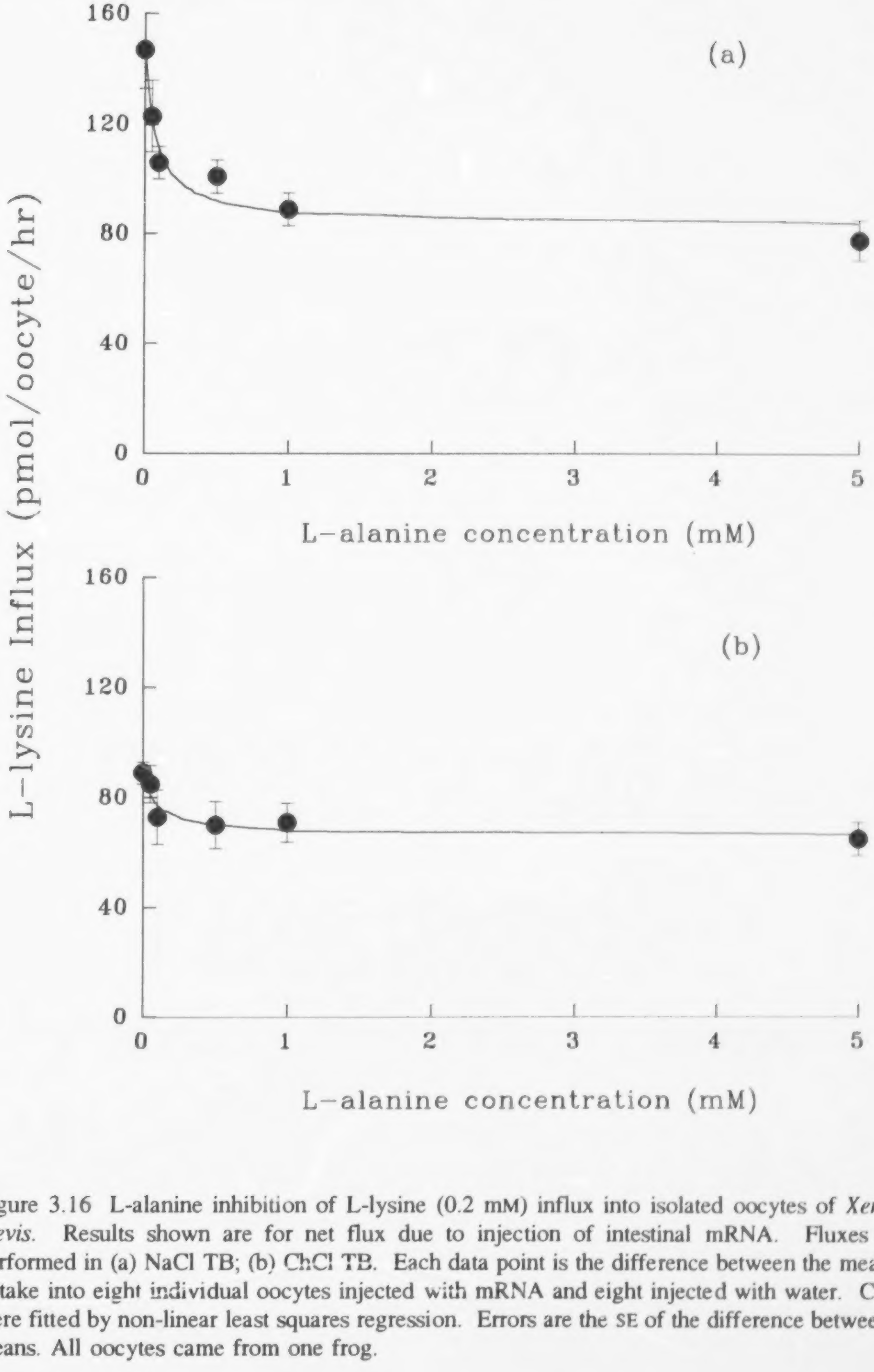

Figure 3.16 L-alanine inhibition of L-lysine (0.2 mM) influx into isolated oocytes of *Xenopus laevis*. Results shown are for net flux due to injection of intestinal mRNA. Fluxes were performed in (a) NaCl TB; (b) ChCl TB. Each data point is the difference between the means of uptake into eight individual oocytes injected with mRNA and eight injected with water. Curves were fitted by non-linear least squares regression. Errors are the SE of the difference between the means. All oocytes came from one frog.

and absence of Na^+. The results of these inhibition experiments are shown in Table 2.1 MeAIB (methyl-amino isobutyric acid - substrate of System A which is a Na^+-dependent transport system specific for small neutral amino acids like L-alanine) did not inhibit the net expressed lysine influx significantly. L-threonine inhibited the net lysine influx by 22% in the presence of Na^+, but had less inhibitory effect in the absence of Na^+. Substrates specific to system $B^{o,+}$(BCH,BCO) and system $b^{o,+}$(valine) were tested as well. The results show a small but significant inhibitory effect of 35% by BCH in the presence of Na^+ where as BCO did not inhibit in either the presence or absence of Na^+. L-valine inhibited lysine influx by 25% in the absence of Na^+. However, when comparing the inhibitory effects of these amino acids to those observed by L-leucine and L-arginine at 1 mM, it suggests that only leucine and arginine in the presence of Na^+ are high affinity competitors of lysine transport.

D) 4F2hc AND D2 ANTISENSE HYBRID-DEPLETION EXPERIMENTS

When the rat intestinal mRNA was tested by diagnostic PCR, both the 4F2hc and D2 PCR products were produced from the rat intestinal mRNA (METHODS - Figure 2.2). Therefore, rat intestinal mRNA was positive for both D2 and 4F2hc. The intestinal mRNA was subjected to antisense-hybrid depletion experiments to test whether 4F2hc and/or D2 were responsible for any of the net expressed lysine uptake induced by the injection of rat intestinal mRNA. In Figure 3.18 addition of the D2 antisense caused a 32 ± 9% and 25 ± 10% reduction in the controlled uptake in the presence and absence of Na^+. The addition of the 4F2hc antisense reduced the control uptake by 25 ± 9% and 41 ± 11% in NaCl and ChCl media respectively. If both 4F2hc and D2 antisense were added together there was no further reduction in the net expressed lysine uptake (data not shown).

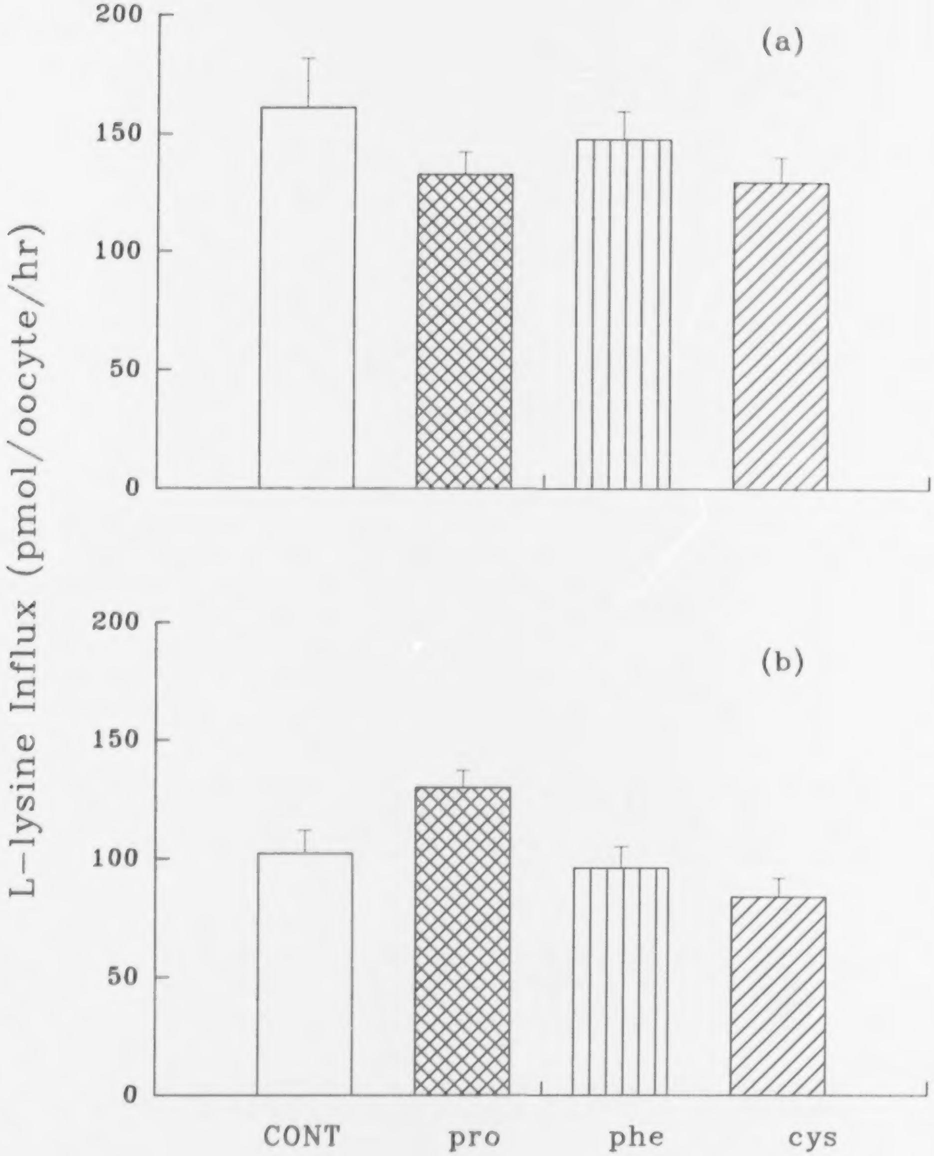

Figure 3.17 Inhibition of L-lysine (0.2 mM) influx into isolated oocytes of *Xenopus laevis* by L-proline (pro), L-phenylalanine, (phe) and cysteine (cys) at 1mM. Results shown are for net flux due to injection of intestinal mRNA. Fluxes were performed in (a) NaCl TB and (b) ChCl TB. Incubations in the presence of L-cysteine also included 1 mM dithiothreitol to prevent oxidation. Each data point is the difference between the means of uptake into eight individual oocytes injected with mRNA and eight oocytes injected with water. Errors are the SE of the difference between the means. All oocytes are from one frog.

Table 2.1 Inhibition of 0.2 mM Lysine Influx into *Xenopus* oocytes by different amino acids.

Inhibitor	NaCl	ChCl
Control	100%	100%
1 mM L-valine	90.0 ± 6.3%	75.1 ± 8.1% *
1 mM L-threonine	78.0 ± 7.8% *	88.7 ± 8.0%
1 mM MeAIB	88.3 ± 10.9%	94.4 ± 8.3%
1 mM BCO	105 ± 6.9%	93.3 ± 5.6%
1 mM BCH	68.3 ± 11.9% *	95.2 ± 8.7%

Results shown are for the net flux due to injection of intestinal mRNA. Inhibition is represented in % of the control flux and was carrried out both in NaCl and ChCl media. The error is the % standard error of difference between the means. Significant differences relative to the control were determined by analysis of variance (ANOVA) and are indicated by * at $p=0.05$

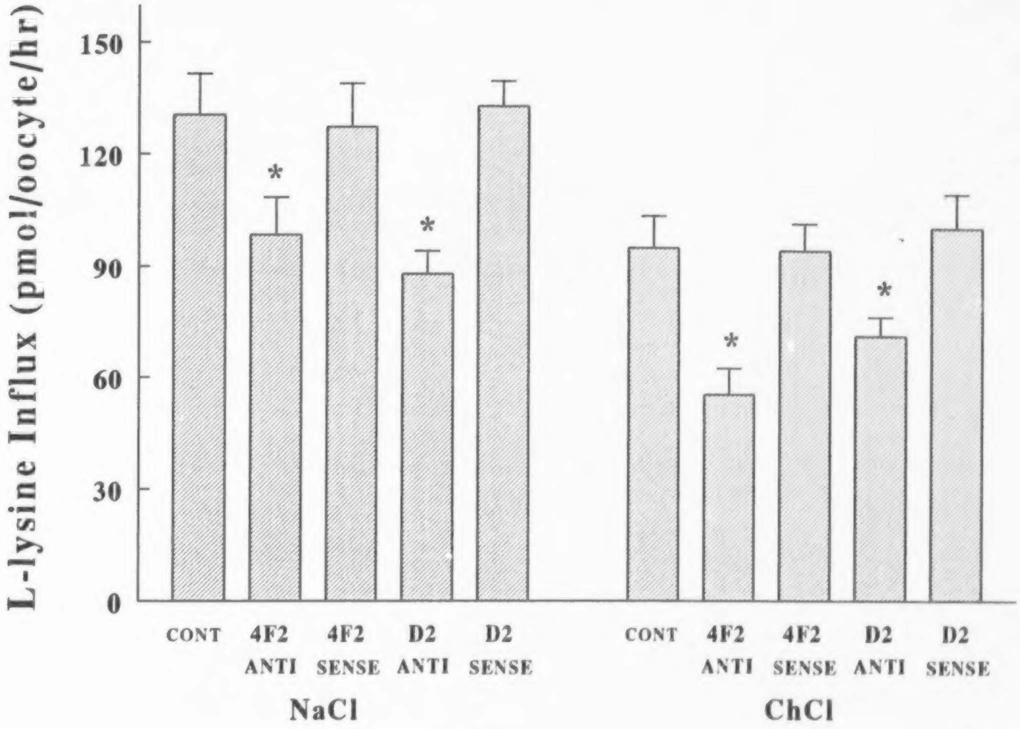

Figure 3.18 4F2 and D2 antisense/hybrid depletion of the intestinal mRNA induced L-lysine (0.2 mM) influx into isolated oocytes of *Xenopus laevis*. For this antisense hybrid deletion experiment either 4F2 antisense/sense or D2 antisense/sense was added to the intestinal mRNA as described in METHODS at a 1:4 ratio. Each data point is the difference between the means of uptake into ten individual oocytes injected with mRNA and ten oocytes injected with water. Errors are the SE of the difference between the means. Significant differences were relative to the control and determined by analysis of variance and are indicated by * ($p=0.05$). All oocytes are from one frog.

E) INTESTINAL mRNA SIZE FRACTIONATION

In order to estimate the size of the intestinal mRNA responsible for exogenous transport of lysine, we size-fractionated mRNA on a 5-25% sucrose density gradient. Samples of every third fraction collected were run on a 1% denaturing agarose gel with ethidium bromide, together with size markers, so that fractions could be chosen for injection (Figure 3.19). The gel was scanned by laser densitometry to give accurate estimates of the size ranges for each fraction. Pooled mRNA fractions, representing size ranges of (A) 2.0 to 2.8, (median 2.6); (B) 1.5 to 2.25 (median 2) ; (C) 0.75 to 1.6, (median 1.1) Kbases, were injected into oocytes and lysine influx (0.2 mM) was measured in NaCl TB with or without 10 mM L-arginine. The results of a representative experiment are shown in Figure 3.20. Some expression of transport activity was found in each of the three fractions tested, and maximum expression was exhibited by fraction B, which corresponds to a size range of 1.5 to 2.25 Kb pairs.

3) CLONING STUDIES

A) CLONING OF RAT INTESTINAL 4F2hc

The cDNA library that was constructed using the pGEM-3Z vector (Promega) and a mRNA size fraction (1.6-3.0 Kb; median 2.3 Kb) was tested for L-lysine transport activity (Figure 3.21). cRNA, made from the cDNA of the total library and injected into oocytes, induced lysine influx of 267 ± 20.3 pmol/oocyte/hr and 252 ± 19.8 pmol/oocyte/hr above the water injected controls. This graph also shows that the net lysine uptake was almost completely Na^+-independent. In a preliminary study, the cDNA library was divided into 40 pools (#1-#40) of 200-300 clones/pool and screened for lysine transport activity. This resulted in very low expression of lysine transport in

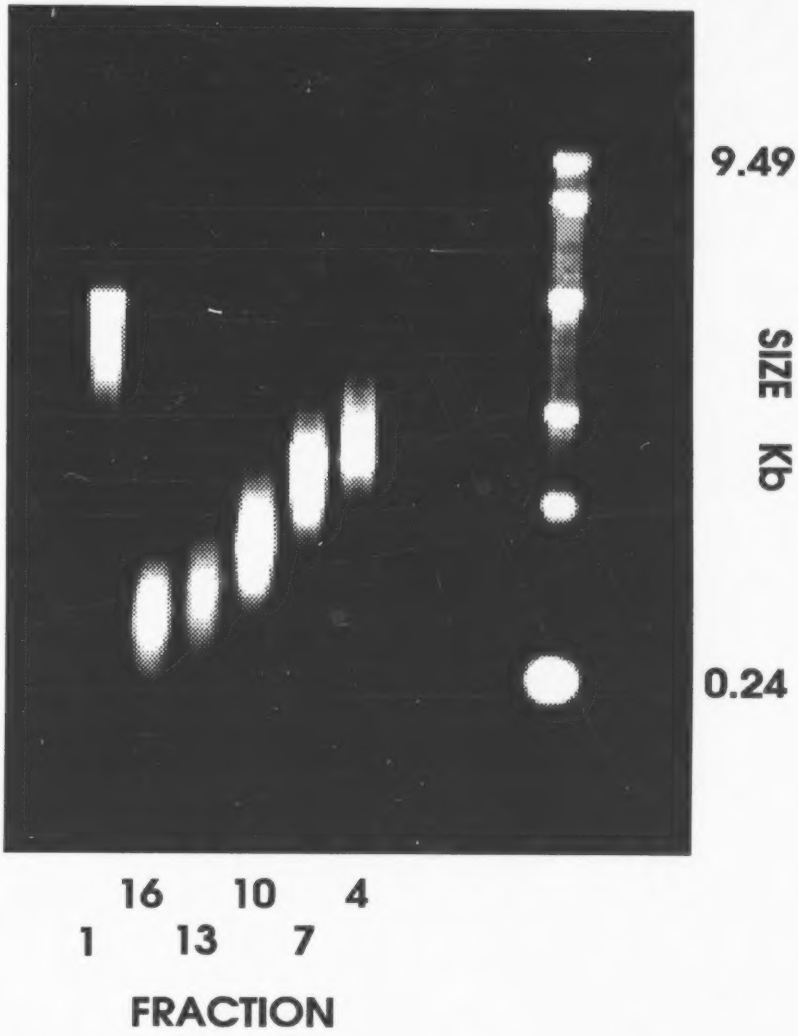

Figure 3.19 A denaturing-agarose gel (1% w/v, 0.66 M formaldehyde) of intestinal mRNA fractions. A 150 μg sample of rat intestinal mRNA was size fractionated on a 5-25% (w/v) sucrose density gradient. Samples of every third fraction were denatured (65° C for 10 min., rapid cooling on ice) and loaded onto the gel with 1 μg ethidium bromide. The gel was run at 40V, 11.5 hr at room temperature, with BRL size markers: 0.24, 1.35, 2.37, 4.4, 7.46, 9.49 kilobases.

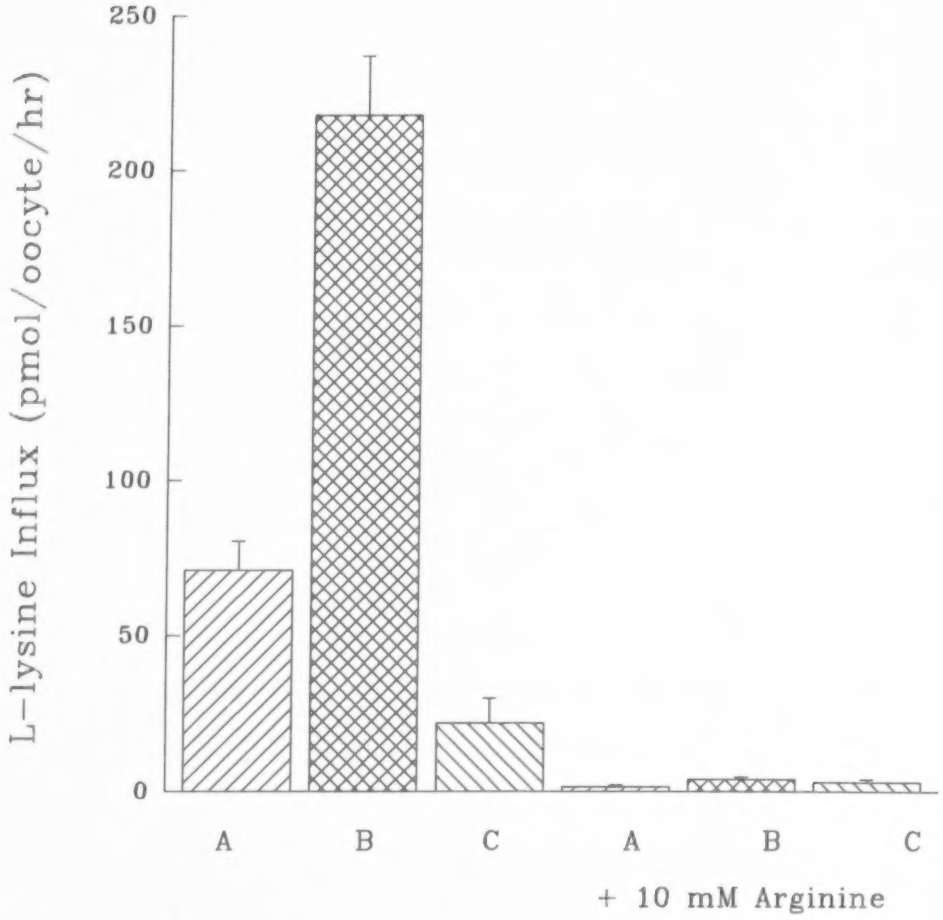

Figure 3.20 Influx of L-lysine (0.2 mM) into isolated oocytes of *Xenopus laevis* injected with different mRNA size fractions. Results shown are for net flux due to injection of fractions, corresponding to those in Figure 3.19 as follows: Fraction A was fractions 4 and 5; fraction B was fractions 6 and 7; fraction C was fractions 8 and 9. Fluxes were performed in NaCl TB in the absence or presence of excess unlabelled (10 mM) L-arginine. Each data point is the difference between the means of uptake into ten individual oocytes injected with an mRNA fraction and ten injected with water. Errors are the SE of the difference of the means. All oocytes are from one frog.

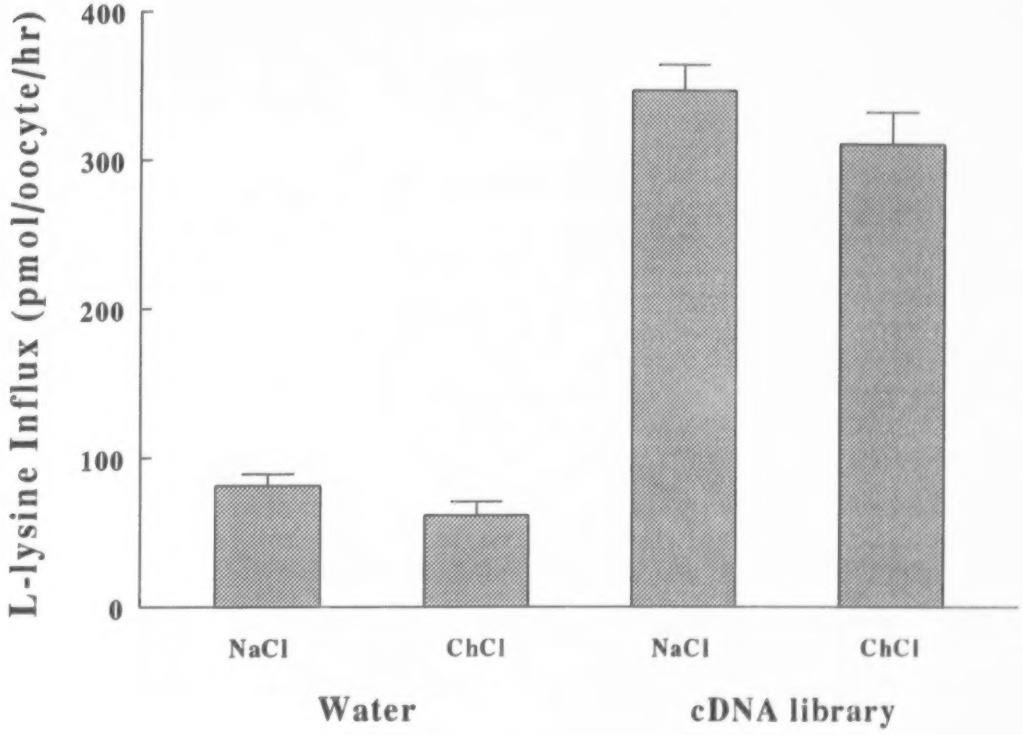

Figure 3.21 Influx of L-lysine (0.2 mM) into isolated oocytes of *Xenopus laevis* due to the injection of cRNA made from the total cDNA library. The oocytes were injected with either water (control) or cRNA made from *in vitro* transcription of cDNA from the total library. Fluxes were performed in either NaCl or ChCl TB. Each data point is the mean of uptake into ten individual oocytes injected with cRNA and for the control, the mean uptake into ten oocytes injected with water. Errors are the SE of the mean. All oocytes came from one frog.

several of the 40 pools (data not shown). The cDNA library was screened again, by subdivision into 22 pools (#41-#62) of 700-800 clones/pool and this resulted in the identification of 4 positive pools (Pool #44, 45, 47, 56) all of which were also positive for 4F2hc and not D2 (Figure 3.22). Pool #56 had the highest lysine influx which was consistently 9-10 fold higher than the water injected controls. As well, pool #47 showed a 7-8 fold increase in lysine influx compared to the control flux. Both pools had a greater lysine transport activity than the level of expression obtained with the total cDNA library (see Figure 3.21). The individual colonies from pool #56 and #47 were seeded into microtitre plates (See METHODS SEC 3B). The rows and columns of pool #56 were tested on three occasions for lysine transport activity with no activity being detected. Following the unsuccessful screening of pool #56, efforts turned to the screening of the rows and columns of pool #47. Out of the 12 columns (#1→#12) and 15 rows (designated A→0 - in which each "row" consisted of a group of 4 rows with respect to the 96-well microtitre plates), one row (Row C) and one column (Column #8) was found that expressed lysine transport activity (Figure 3.23). Each row from Pool #47 consisted of 4 rows and 12 columns. From Row C, columns #8 and row A^* showed a 4-5 fold increase in lysine transport compared to the water injected controls (Figure 3.24). Testing the rows and columns for 4F2hc activity by using the diagnostic PCR technique revealed that both column 8 and row A^* were both positive for 4F2hc (Figure 3.25). The cRNA from well 8A gave a 4-5 fold increase in lysine uptake (Figure 3.26) and diagnostic PCR determined that the single clone from well 8A was related to the 4F2hc glycoprotein, but not to the D2 glycoprotein (Figure 3.25). The 4F2 clone itself induced a lower lysine transport activity than that induced by the cRNA from pool #47. Possible explanations for this drop in activity are outlined and discussed in the Chapter V (DISCUSSION).

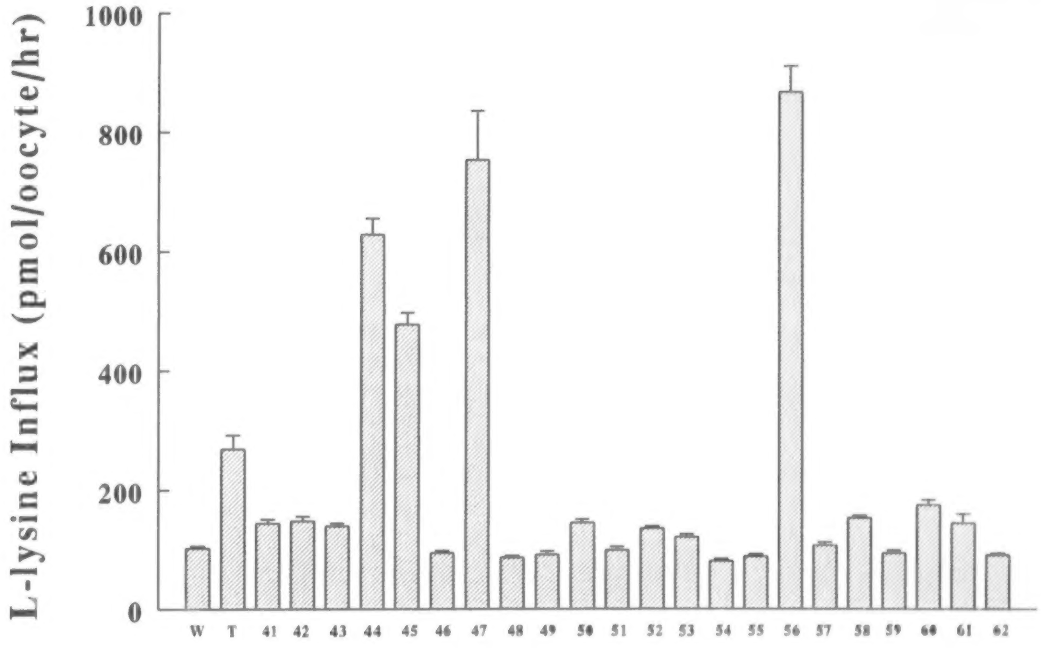

Figure 3.22 Screening of cDNA library pools for L-lysine (0.2 mM) activity. The cDNA library was subdivided into 22 pools (#41-#62) and cDNA from each pool was *in vitro* transcribed to cRNA and injected into oocytes. The total cDNA library is represented by (T) and the water-injected controls is represented by (W). Fluxes were performed in NaCl TB. Each data point is the mean of uptake into ten individual oocytes injected with cRNA and for the control, the mean of uptake into ten oocytes injected with water. Errors are the SE of the mean. All oocytes came from one frog.

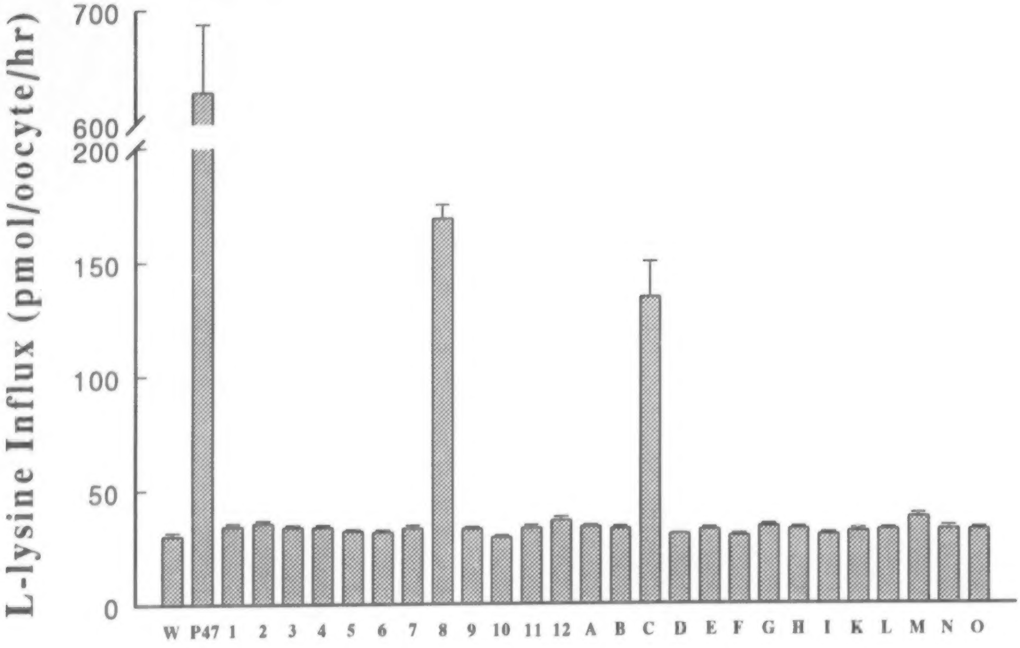

Figure 3.23 Screening of rows and columns of Pool#47 for L-lysine (0.2 mM) transport activity. Pool #47 tested positive for lysine transport activity and was further divided into 12 columns (#1-#12) and 15 rows (A→O) producing a grid system. The cDNA from each row or column was *in vitro* transcribed to cRNA and injected into oocytes. The water injected controls are represented by (W) and Pool #47 is designated (P47). Fluxes were performed in NaCl TB. Each data point is the mean of uptake into ten individual oocytes injected with cRNA and for the control, the mean of uptake into ten oocytes injected with water. Errors are the SE of the mean. All oocytes came from one frog.

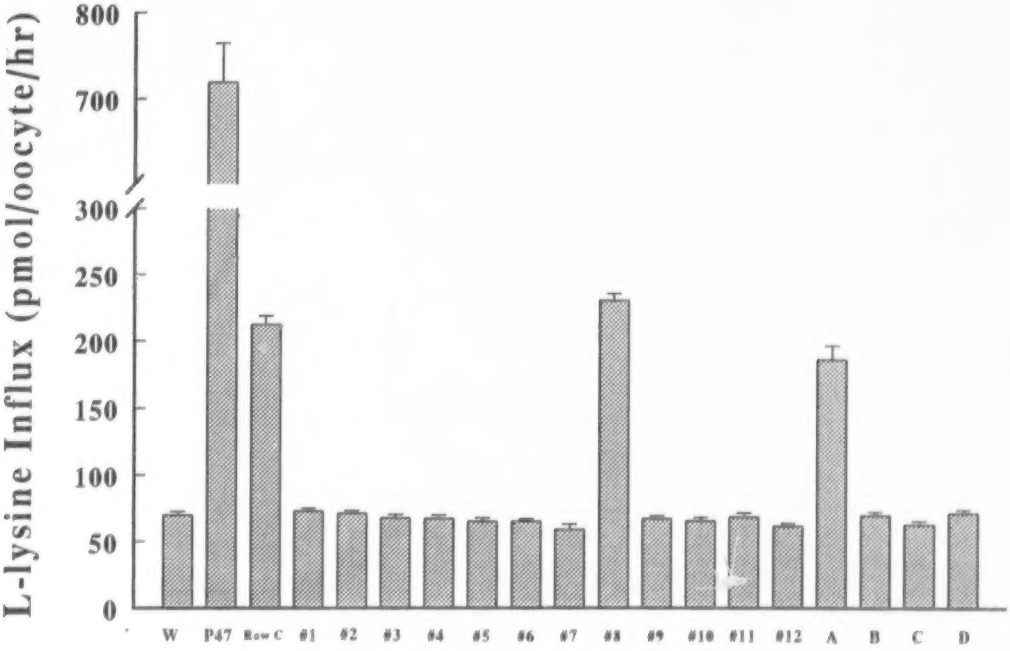

Figure 3.24 Screening of the rows and columns from Row C of Pool #47. The corresponding positive Row C from Figure 3.23 was subdivided into 12 columns (#1-#12) and 4 rows (A→D) producing a grid system. The cDNA from each row or column was *in vitro* transcribed to cRNA and injected into oocytes. The water injected controls are represented by (W), Pool #47 is designated (P47), and Row C from Pool #47 is designated (Row C). Fluxes were performed in NaCl TB. Each data point is the mean of uptake into ten individual oocytes injected with cRNA or for the control, the mean of uptake into ten oocytes injected with water. Errors are the SE of the mean. All oocytes came from one frog.

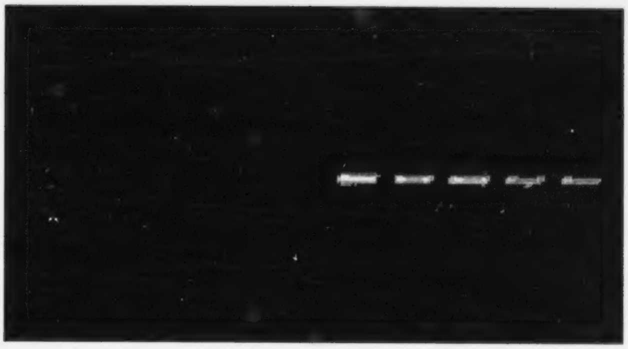

Figure 3.25 An agarose gel (2%(w/v)) of the PCR products obtained from cDNA from the following: Well 8A (lane 1,6); Row A (lane 2,7); Column 8 (lane 3,8); Row C from Pool #47 (lane 4,9); Pool#47 (lane 5,10). Lanes 1-5 were subjected to diagnostic PCR using D2 primers; Lane 6-10 were subjected to diagnostic PCR using 4F2 primers.

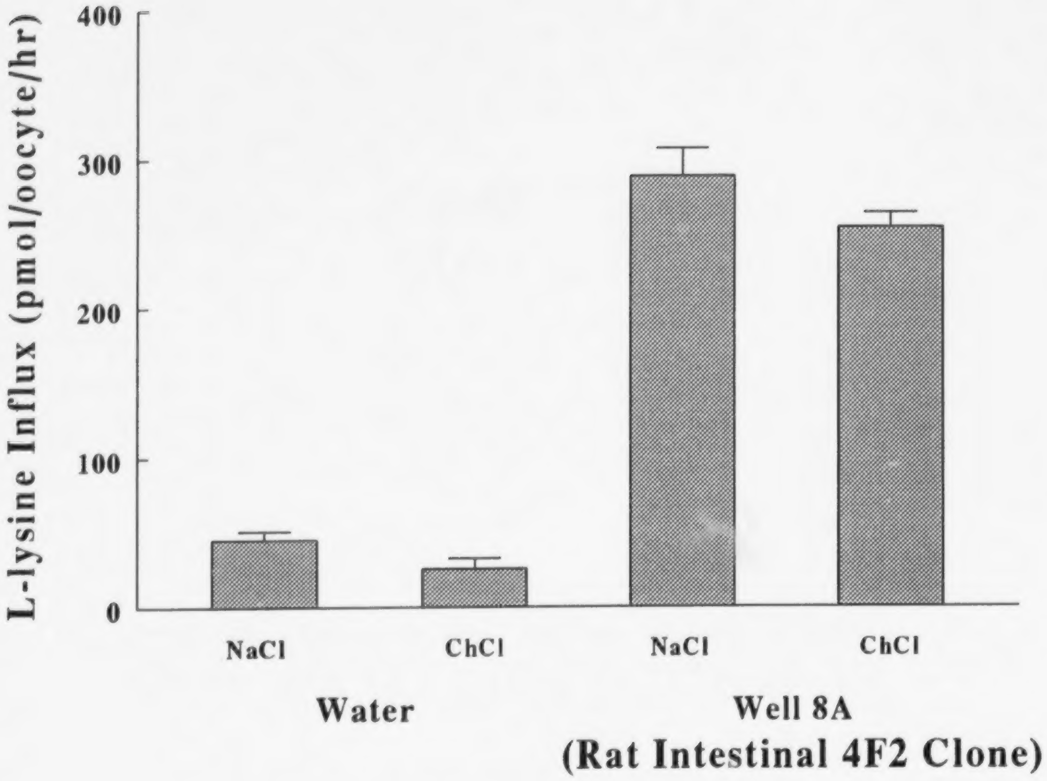

Figure 3.26 Influx of L-lysine (0.2 mM) into isolated oocytes of *Xenopus laevis*. The oocytes were injected with either water (control) or cDNA from well 8A (rat intestinal 4F2). Fluxes were performed in either NaCl or ChCl TB. Each data point is the mean of uptake into ten individual oocytes injected with cRNA and for the control, the mean uptake into ten oocytes injected with water. Errors are the SE of the mean. All oocytes came from one frog.

B) THE RAT INTESTINAL 4F2hc NUCLEOTIDE & DEDUCED AMINO ACID SEQUENCES

The rat intestinal 4F2hc cDNA insert was 1.8 Kb in length, with an open reading frame encoding a protein of 527 amino acids (Figure 3.27). When compared to amino acid sequence of mouse fibroblast 4F2hc there was 89% amino acid identity and 93% similarity (Figure 3.28). Rat intestinal 4F2hc protein is also homologous to the human fibroblast isoform having 76% amino acid identity and 86% similarity (Figure 3.29). The 4F2hc protein is part of a larger family of membrane glycoproteins that includes rBAT (D2) known to induce cystine and dibasic amino acid transport in *Xenopus* oocytes. and has been implicated in the human intestinal/kidney amino acid transport disorder, cystinuria [19,135]. This family of proteins share a similar secondary structure in that they contain only one transmembrane domain. A hydropathy analysis of rat intestinal 4F2hc is shown in Figure 3.30 and portrays a single potential hydrophobic transmembrane region near the N-terminus at amino acid residues 75-99.

C) CHARACTERIZATION OF 4F2hc CLONE

The K_m and V_{max} were determined for the net expressed lysine influx induced by rat intestinal 4F2hc. In the presence of Na^+, the K_m was 0.046 ± 0.016 mM and the Vmax was 300 ± 22 pmol/oocyte/hr. In the absence of Na^+, the K_m was 0.044 ± 0.006 mM and Vmax was 243 ± 8.5 pmol/oocyte/hr (Figure 3.31). Competition with other amino acids resulted in significant inhibition of the net lysine influx by L-arginine, L-leucine, L-alanine, L-phenylalanine, and L-valine (Figure 3.32). Complete inhibition was obtained with L-arginine and L-leucine at 10 mM in the presence of Na^+. In the absence of Na^+, L-arginine also exhibited complete inhibition, however, L-leucine inhibition was reduced to 25 ± 7 %. Both L-valine and L-phenylalanine inhibition were also reduced to around 25-30% in the ChCl media. L-valine had no inhibitory effect when NaCl was

```
                                              CCTCACGGGACACCT 15
CTCCAAACTAGGCGACCCACTGACTACAGGCCCGTGTCCTGTCACCGTTTCTGCAGGTACC 75
ATCAGCCAGGACACCGAAGTGGACATGAAAGATGTGGAGCTGAACGAGCTGGAACCGGAG 135
M  S  Q  D  T  E  V  D  M  K  D  V  E  L  N  E  L  E  P  E  20

AAGCAGCCTATGAATGCAGCGGACGGGGCGGCCGCCGGGGAGAAGAACGGTCTGGTGAAG 195
K  Q  P  M  N  A  A  D  G  A  A  A  G  E  K  N  G  L  V  K  40

ATTAAGGTGGCCGAAGACGAGGCGGAAGCCGGGGTCAAGTTCACAGGCTTATCCAAGGAG 255
I  K  V  A  E  D  E  A  E  A  G  V  K  F  T  G  L  S  K  E  60

GAGCTATTGAAGGTAGCTGGCAGCCCGGGCTGGGTGCGCACCCGCTGGGCGCTGCTGCTG 315
E  L  L  K  V  A  G  S  P  G  W  V  R  T  R  W  A  L  L  L  80

CTCTTCTGGCTCGGTTGGCTGGGTATGCTGGCGGGCGCCGTGGTTATCATCGTTCGGGCG 375
L  F  W  L  G  W  L  G  M  L  A  G  A  V  V  I  I  V  R  A  100

CCACGCTGCCGTGAGCTGCCGGTACAGAGATGGTGGCACAAGGGCGCCCTCTACCGCATC 435
P  R  C  R  E  L  P  V  Q  R  W  W  H  K  G  A  L  Y  R  I  120

GGCGACCTTCAGGCCTTCGTAGGCCCGGAAGCGAGAGGCATAGCTGGTCTGAAGAACCAT 495
G  D  L  Q  A  F  V  G  P  E  A  R  G  I  A  G  L  K  N  H  140

CTGGAGTACTTGAGCACCCTGAAGGTGAAGGGCCTAGTTTTGGGCCCAATTCACAAGAAC 555
L  E  Y  L  S  T  L  K  V  K  G  L  V  L  G  P  I  H  K  N  160

CAGAAGGATGAAGTCAATGAAACCGACTTGAAACAGATTGATCCCGATTTAGGCTCCCAG 615
Q  K  D  E  V  N  E  T  D  L  K  Q  I  D  P  D  L  G  S  Q  180

GAAGATTTTAAAGACCTTCTACAAAGTGCCAAGAAAAAGAGCATTCACATCATTTTGGAC 675
E  D  F  K  D  L  L  Q  S  A  K  K  K  S  I  H  I  I  L  D  200

CTCACTCCCAACTATAAGGGCCAGAATGCATGGTTCCTCCCTCCTCAGGCTGACATTGTA 735
L  T  P  N  Y  K  G  Q  N  A  W  F  L  P  P  Q  A  D  I  V  220

GCCACCAAAATGAAGGAGGCTCTGAGTTCTTGGTTGCAGGACGGTGTGGATGGGTTCCAA 795
A  T  K  M  K  E  A  L  S  S  W  L  Q  D  G  V  D  G  F  Q  240

GTTCGGGATGTGGGAAAGCTGGCGAATGCATCCTTGTACTTGGCTGAGTGGCAGAATATC 855
V  R  D  V  G  K  L  A  N  A  S  L  Y  L  A  E  W  Q  N  I  260

ACCAAGAACTTCAGTGAGGACAGGCTTTTGATTGCAGGGACCGCGTCCTCTGACCTGCAA 915
T  K  N  F  S  E  D  R  L  L  I  A  G  T  A  S  S  D  L  Q  280

CAAATTGTCAACATACTTGAATCCACCAGCGATCTGCTGCTGACCAGCTCATACCTGTCA 975
Q  I  V  N  I  L  E  S  T  S  D  L  L  L  T  S  S  Y  L  S  300

CAGCCCGTTTTCACTGGGGAGCATGCAGAACTCCTAGTGATTAAGTATTTGAATGCCACT 1035
Q  P  V  F  T  G  E  H  A  E  L  L  V  I  K  Y  L  N  A  T  320

GGCAGCCGCTGGTGCAGCTGGAGTGTGTCGCAGGCAGGACTCCTGACATCCTTTATACCG 1095
G  S  R  W  C  S  W  S  V  S  Q  A  G  L  L  T  S  F  I  P  340

GATCAGTTTCTCCGACTCTACCAGCTGCTGCTCTTCACTCTGCCAGGAACTCCTGTTTTC 1155
D  Q  F  L  R  L  Y  Q  L  L  L  F  T  L  P  G  T  P  V  F  360

AGCTATGGGGATGAGCTTGGCCTTCAGGCAGTTGCCCTTCCTGGACAGCCTATGGAGGCT 1215
S  Y  G  D  E  L  G  L  Q  A  V  A  L  P  G  Q  P  M  E  A  380

CCATTCATGCTGTGGAATGAGTCTAGCAACTCCCAAACCTCAAGTCCTGTAAGCCTCAAC 1275
P  F  M  L  W  N  E  S  S  N  S  Q  T  S  S  P  V  S  L  N  400

ATGACAGTGAAGGGCCAAAATGAAGACCCCGGCTCCCTCCTCACCCAGTTCCGGCGACTG 1335
M  T  V  K  G  Q  N  E  D  P  G  S  L  L  T  Q  F  R  R  L  420

AGTGACCTCCGTGGTAAGGAGCGCTCTCTGTTACACGGTGACTTTGATGCACTGTCTTCC 1395
S  D  L  R  G  K  E  R  S  L  L  H  G  D  F  D  A  L  S  S  440

TCATCTGGGCTCTTCTCCTACGTCCGCCACTGGGACCAGAATGAGCGTTACCTGGTGGTG 1455
S  S  G  L  F  S  Y  V  R  H  W  D  Q  N  E  R  Y  L  V  V  460

CTCAACTTCCAGGATGTGGGCCTGTCAGCCAGGGTAGGAGCCTCCAACCTCCCTGCTGGC 1515
L  N  F  Q  D  V  G  L  S  A  R  V  G  A  S  N  L  P  A  G  480

ATAAGCCTGCCAGCCAGTGCTAACCTTTTGCTTAGTACTGACAGCACCCGGCTAAACCGT 1575
I  S  L  P  A  S  A  N  L  L  L  S  T  D  S  T  R  L  N  R  500

GAGGAGGGCACCTCCCTGAGCCTGGAAAACCTGAGCCTGAAGCCGGATGAGGGCTTGTTG 1635
E  E  G  T  S  L  S  L  E  N  L  S  L  K  P  D  E  G  L  L  520

TTACAGTTCCCTTTTGTGGCCTGATCCCTCTACACAGAACCTGCCACCCTTCTTTCCTCT 1695
L  Q  F  P  F  V  A  *                                      527

CTCAGGCCTTTGGAAATCTGGTCTTTCTCTCCTTATTTTGTTTTTGTTTTTAAACTTTTG 1755
```

Figure 3.27 The nucleotide and amino acid sequence of rat intestinal 4F2hc. * denotes the stop condon (TGA) and the underlined residues are the two cysteine amino acids.

```
                                    .         .         .         .         .
Mouse fibroblast 4F2hc   1 MSQDTEVDMKDVELNELEPEKQPMNAADGAAAGEKNGLVKIKVAEDETEA 50
                           |||||||||||||||||||||||||||||||||||||||||||||||.||
Rat intestinal 4F2hc     1 MSQDTEVDMKDVELNELEPEKQPMNAADGAAAGEKNGLVKIKVAEDEAEA 50
                                    .         .         .         .         .
                        51 GVKFTGLSKEELLKVAGSPGWVRTRWALLLLFWLGWLGMLAGAVVIIVRA 100
                           ||||||||||||||||||||||||||||||||||||||||||||||||||
                        51 GVKFTGLSKEELLKVAGSPGWVRTRWALLLLFWLGWLGMLAGAVVIIVRA 100
                                    .         .         .         .         .
                       101 PRCRELPVQRWWHKGALYRIGDLQAFVGRDAGGIAGLKSHLEYLSTLKVK 150
                           ||||||||||||||||||||||||||||.:| ||||||.|||||||||||
                       101 PRCRELPVQRWWHKGALYRIGDLQAFVGPEARGIAGLKNHLEYLSTLKVK 150
                                    .         .         .         .         .
                       151 GLVLGPIHKNQKDEINETDLKQINPTLGSQEDFKDLLQSAKKKSIHIILD 200
                           ||||||||||||||:||||||||:|.||||||||||||||||||||||||
                       151 GLVLGPIHKNQKDEVNETDLKQIDPDLGSQEDFKDLLQSAKKKSIHIILD 200
                                    .         .         .         .         .
                       201 LTPNYQGQNAWFLPAQADIVATKMKEALSSWLQDGVDGFQFRDVGKLMNA 250
                           |||||.||||||||:|||||||||||||||||||||||||.|||||| ||
                       201 LTPNYKGQNAWFLPPQADIVATKMKEALSSWLQDGVDGFQVRDVGKLANA 250
                                    .         .         .         .         .
                       251 PLYLAEWQNITKNLSEDRLLIAGTESSDLQQIVNILESTSDLLLTSSYLS 300
                           .||||||||||||:||||||||||.|||||||||||||||||||||||||
                       251 SLYLAEWQNITKNFSEDRLLIAGTASSDLQQIVNILESTSDLLLTSSYLS 300
                                    .         .         .         .         .
                       301 NSTFTGERTESLVTRFLNATGSQWCSWSVSQAGLLADFIPDHLLRLYQLL 350
                           ...||||:.| ||.::||||||.||||||||||||..||||::|||||||
                       301 QPVFTGEHAELLVIKYLNATGSRWCSWSVSQAGLLTSFIPDQFLRLYQLL 350
                                    .         .         .         .         .
                       351 LFTLPGTPVFSYGDELGLQG.ALPGQPAKAPLMPWNESSIFHIPRPVSLN 399
                           |||||||||||||||||||: |||||| .||:| |||||  :...|||||
                       351 LFTLPGTPVFSYGDELGLQAVALPGQPMEAPFMLWNESSNSQTSSPVSLN 400
                                    .         .         .         .         .
                       400 MTVKGQNEDPGSLLTQFRRLSDLRGKERSLLHGDFHALSSSPDLFSYIRH 449
                           |||||||||||||||||||||||||||||||||||.|||||.:||||:||
                       401 MTVKGQNEDPGSLLTQFRRLSDLRGKERSLLHGDFDALSSSSGLFSYVRH 450
                                    .         .         .         .         .
                       450 WDQNERYLVVLNFRDSGRSARLGASNLPAGISLPASAKLLLSTDSARQSR 499
                           |||||||||||||.| | |||:|||||||||||||||.|||||||.| .|
                       451 WDQNERYLVVLNFQDVGLSARVGASNLPAGISLPASANLLLSTDSTRLNR 500
                                    .         .
                       500 EEDTSLKLENLSLNPYEGLLLQFPFVA 526
                           ||:|||.||||||.| |||||||||||
                       501 EEGTSLSLENLSLKPDEGLLLQFPFVA 527
```

Figure 3.28 Alignment of the mouse fibroblast 4F2hc and rat intestinal 4F2hc sequences. The symbols between amino acids from the two sequences represent the following: (|) - identical; (:) - highly conserved; (·) - conserved; (no symbol) - not conserved.

```
                                    .         .         .         .         .
Human 4F2hc                1 MSQDTEVDMKEVELNELEPEKQPMNAASGAAMSLAEAEKNGLVKIKVAED 50
                             ||||||||||:||||||||||||||||.||    |.:|||||||||||||
Rat intestinal 4F2hc       1 MSQDTEVDMKDVELNELEPEKQPMNAADGA....AAGEKNGLVKIKVAED 46

                                    .         .         .         .         .
                          51 EAEAAAAAKFTGLSKEELLKVAGSPGWVRTRWALLLLFWLGWLGMLAGAV 100
                             |||  |:.||||||||||||||||||||||||||||||||||||||||||
                          47 EAE..AGVKFTGLSKEELLKVAGSPGWVRTRWALLLLFWLGWLGMLAGAV 94

                                    .         .         .         .         .
                         101 VIIVRAPRCRELPAQKWWHTGPLYRIGDLQAFQGHGAGNLAGLKGRLDYL 150
                             |||||||||||||.|:|||.|:|||||||||| |.:| .:||||.:|:||
                          95 VIIVRAPRCRELPVQRWWHKGALYRIGDLQAFVGPEARGIAGLKNHLEYL 144

                                    .         .         .         .         .
                         151 SSLKVKGLVLGPIHKNQKDDVAQTDLLQIDPNFGSKEDFDSLLQSAKKKS 200
                             |.|||||||||||||||||:|.:||| ||||::||.|||..|||||||||
                         145 STLKVKGLVLGPIHKNQKDEVNETDLKQIDPDLGSQEDFKDLLQSAKKKS 194

                                    .         .         .         .         .
                         201 IRVILDLTPNYRGDNSWF.STQVDTVATKVKDALEFWLQAGVDGFQVRDI 249
                             |::||||||||:|:|.|| ..|.|.||||:|:||. |||.|||||||||:
                         195 IHIILDLTPNYKGQNAWFLPPQADIVATKMKEALSSWLQDGVDGFQVRDV 244

                                    .         .         .         .         .
                         250 ENLKDASSFLAEWQNITKGFSGDRLLIAGTNSSDLQQILSLLESNKDLLL 299
                             :.| :|| :|||||||||.||:||||||||.|||||||:.:|||..||||
                         245 GKLANASLYLAEWQNITKNFSEDRLLIAGTASSDLQQIVNILESTSDLLL 294

                                    .         .         .         .         .
                         300 TSSYLSDSGSTPQHTKSLVTQYLNATGNRWCSWSLSQARLLTSFLPAQLL 349
                             ||||||:.. |.:|.. ||..||||||.||||||:||| |||||:|.|:|
                         295 TSSYLSQPVFTGEHAELLVIKYLNATGSRWCSWSVSQAGLLTSFIPDQFL 344

                                    .         .         .         .         .
                         350 RLYQLMLFTLPGTPLFSYGDEIGLDAAALPPQPMEAPVMLWDESSFPDIP 399
                             |||||:||||||||:||||||:||:|.|||.||||||.|||:||| .:..
                         345 RLYQLLLFTLPGTPVFSYGDELGLQAVALPGQPMEAPFMLWNESSNSQTS 394

                                    .         .         .         .         .
                         400 GAVSANMTVKGQSEDPGSLLSLFRRLSDQRSKERSLLHGDFHAFSAGPGL 449
                             ::|| |||||||.|||||||. |||||| |:||||||||||.|:|.:.||
                         395 SPVSLNMTVKGQNEDPGSLLTQFRRLSDLRGKERSLLHGDFDALSSSSGL 444

                                    .         .         .         .         .
                         450 FSYIRHWDQNERFLVVLNFGDVGLSAGLQASDLPASASLPAKADLLLSTQ 499
                             |||:||||||||:||||||.|||||| :.||:|||: ||||.|:|||||:
                         445 FSYVRHWDQNERYLVVLNFQDVGLSARVGASNLPAGISLPASANLLLSTD 494

                                    .         .         .
                         500 P...GREEGSPPELGRLKLEPHEGLLLRFPYAA 529
                             .   .||||.. .|:.|.|.|.|||||.||:.|
                         495 STRLNREEGTSLSLENLSLKPDEGLLLQFPFVA 527
```

Figure 3.29 Alignment of human 4F2hc sequence and rat intestinal 4F2hc sequence. The symbols between amino acids from the two sequences represent the following: (|) - identical; (:) - highly conserved; (·) - conserved; (no symbol) - not conserved.

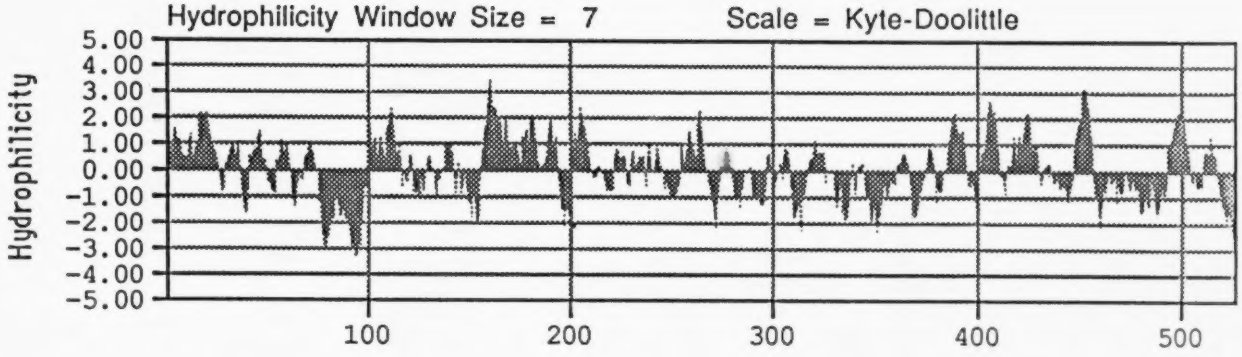

Figure 30.30 Kyte-Doolittle hydropathy analysis of rat intestinal 4F2hc.

replaced by ChCl. These results demonstrate a reduction of the inhibitory effects of neutral amino acids in the absence of Na^+, suggesting a Na^+-dependent interaction with lysine transport.

Since L-leucine had a strong inhibitory effect on the lysine influx induced by rat intestinal 4F2hc, the interaction of these two amino acids was studied further. All previous results suggest that both L-lysine and L-leucine are being transported by the same carrier. To investigate this possibility further efflux experiments were conducted. With radio-labelled lysine preloaded inside oocytes, 5 mM L-leucine was added to the extracellular medium. The results in Figure 3.33 show that L-leucine was able to stimulate efflux of lysine only in the presence of Na^+ and only in the oocytes injected with rat intestinal 4F2hc cRNA. This stimulation of efflux could also be observed using a lower concentration of leucine of 1 mM (data not shown). Subtraction of the endogenous efflux (efflux in the water-injected eggs) from the total efflux, allowed the calculation of the net efflux induced by the injection of intestinal 4F2hc cRNA (exogenous efflux). This is shown in Figure 3.34 and suggests that there was essentially no 4F2hc-induced efflux of [^{3}H] L-lysine until the addition of L-leucine. Therefore, the rate of [^{3}H] L-lysine efflux in Na^+ medium was 4-fold greater then that in ChCl medium. Thus, L-leucine caused Na^+-dependent *trans*-stimulation of L-lysine.

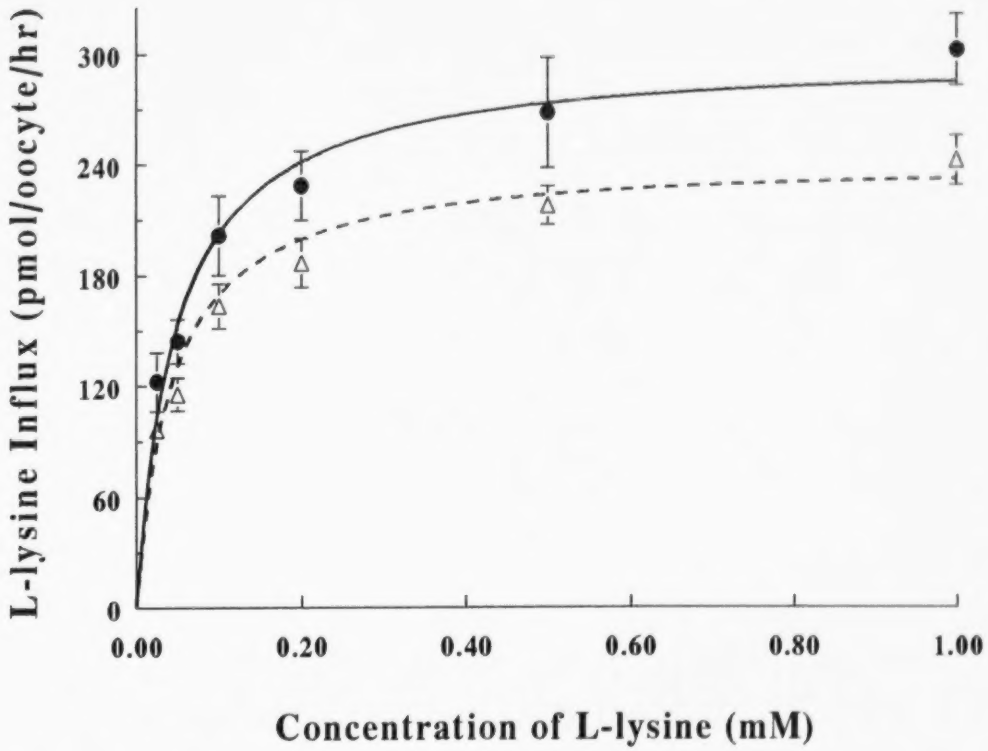

Figure 3.31 Concentration dependence of L-lysine (0.2 mM) in isolated oocytes of *Xenopus laevis* injected with rat intestinal 4F2hc cRNA. Results shown are for net flux due to injection of rat intestinal 4F2hc cRNA. Fluxes were performed in NaCl TB (closed circles) and ChCl TB (open triangles). Each data point is difference between means of uptake into 10 individual oocytes injected with rat intestinal 4F2hc cRNA and 10 individual oocytes injected with water. Curves were fitted by non-linear regression analysis. Errors bars are the SE of difference between the means. All oocytes came from one frog.

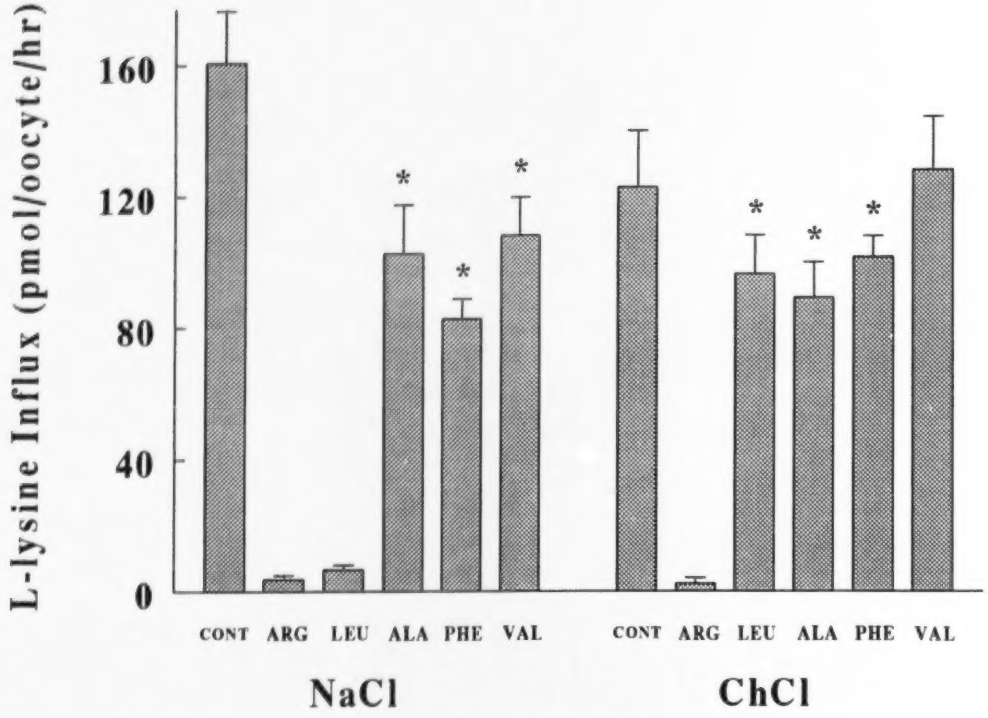

Figure 3.32 Inhibition of L-lysine (0.2 mM) influx into isolated oocytes of *Xenopus laevis* injected with rat intestinal 4F2hc cRNA. Results shown are for net flux due to injection of rat intestinal 4F2hc cRNA. Fluxes were performed in NaCl or ChCl TB in the presence of 10 mM non-radioactive amino acids: L-arginine (ARG); L-leucine (LEU); L-alanine (ALA); L-phenylalanine (PHE); L-valine (VAL). Each data point is difference between means of uptake into 10 individual oocytes injected with rat intestinal 4F2hc cRNA and 10 individual oocytes injected with water. Errors bars are the SE of difference between means. Significant differences were calculated relative to the control (NaCl or ChCl media) and determined by analysis of variance; they are indicated by * (p=0.05). All oocytes came from one frog.

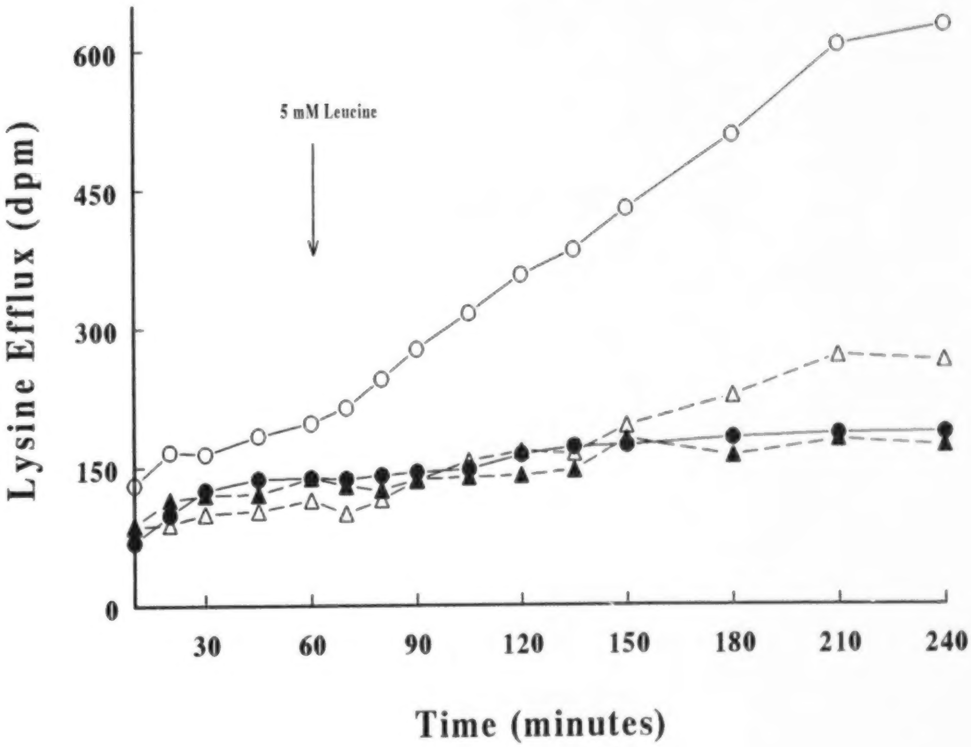

Figure 3.33 Efflux of L-lysine from isolated oocytes of *Xenopus laevis*. Results shown are the efflux of ^{3}H-L-lysine over time from a pool of 25 oocytes. After 60 minutes L-leucine was added in a small volume (5% of total volume), giving a final concentration of 5 mM. Results are for efflux in NaCl TB from with oocytes injected with rat intestinal 4F2hc cRNA (open circles) or with water (closed circles) or efflux in ChCl TB from oocytes injected with rat intestinal 4F2hc cRNA (open triangles) or water (closed triangles). All oocytes came from one frog.

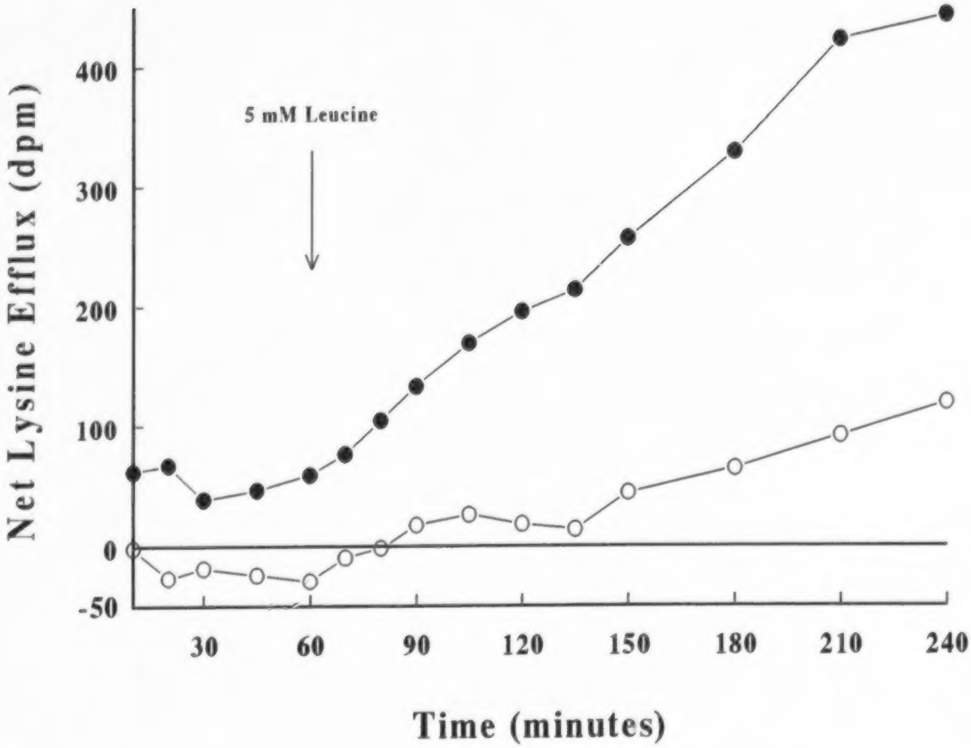

Figure 3.34 Net efflux of L-lysine from of isolated oocytes of *Xenopus laevis*. Results shown are the net efflux of ^{3}H-L-lysine over time from a pool of 25 oocytes. After 60 minutes L-leucine was added in a small volume (5% of total volume), giving a final concentration of 5 mM. Uptake was performed in NaCl TB (closed circles) or ChCl TB (open circles). All oocytes came from one frog.

V. DISCUSSION

A) Brush Border Membrane Lysine Transport

In previous studies, the characterization of lysine transport across the intestinal epithelium has employed techniques such as whole-tissue perfusion, isolated cells and membrane vesicles. In this study everted sleeves were used to look at BBM lysine transport. This preparation overcomes many of the technical problems associated with the other methods mentioned above. Whole-tissue experiments used in earlier studies did not take into account the two independent transport processes occuring at the BBM and BLM [47,82]. Transport was seen as a single process from the lumen of the small intestine into the blood. Later studies using everted sacs observed transport across the BBM, BLM, and subcutaneous layers to the inside of the sac [185]. Isolated cell preparations have not addressed problems of unstirred layers which might potentially lead to an underestimation of V_{max} and an overestimation K_m for transport [72]. Many studies have used BBM vesicles to specifically look at BBM transport for various substrates. However, since lysine is a positively charged amino acid it binds non-specifically to the external membrane of these vesicles [72]. These technical difficulties can be addressed and overcome when everted sleeves are used. First, this preparation specifically measures transport across the BBM into the enterocyte. The potential problem of unstirred layers is overcome by rapidly stirring the incubation media during the uptake period. Non-specific binding still occurs but not to the same extent as in the vesicle preparation. Most of the exterior radiolabelled lysine is removed during a washing step prior to the assaying of intracellular radioactivity. Diffusion of the substrate between cells is accounted and corrected for by measuring PEG uptake (a non-permeable extracellular space marker). Inhibition of the corrected lysine uptake by arginine measures the transportable component of lysine influx and the amount remaining is non-specific binding. From the results in Figure 3.3, binding accounted for less than 20% of the net lysine uptake remaining after inhibition with 5 mM arginine. For these reasons the *in vitro* everted sleeve preparation was used to investigate the transport of L-lysine across the BBM. From the concentration dependence experiment L-lysine transport was shown to occur through relatively high affinity transport systems (see Figure 3.1). Some earlier work had determined the K_m for lysine transport to be in the mM range [86,101,113,117]. However, most studies have reported the K_m values within the μM range [23,150,174,190]. Even Haghira *et al*, the first group to investigate dibasic amino acid transport reported a K_m in the range of 600-700 μM [54]. Therefore, across most species and between most tissues, including the rat intestine as shown in this study, BBM lysine transport has been reported to be *via* high affinity transport systems.

A debate in the literature has arisen as to whether lysine transport exhibits any Na^+-dependence. In rat intestinal BBM vesicles, cationic amino acid transport has been shown to be Na^+-independent [23], but also Na^+-dependent [190]. From the time lysine transport was first investigated, system y^+, a Na^+-independent transporter, specific for dibasic amino acids, has been thought to account for the majority of lysine transport. However, numerous investigators have observed some Na^+-dependence for lysine transport in many diverse tissues [77,116,148,174]. One possible reason for such contrasting results, is the finding that the presence of Na^+-dependence is diet dependent [191]. As alluded to in the Introduction, vesicles from rats fed on a low-protein diet (13% protein) exhibited only Na^+-independent lysine transport, but rats fed on high protein diet (88% protein) showed an additional Na^+-dependent component [191]. The data from everted sleeve experiments used rats fed on a standard diet containing 23% protein and supports the presence of a Na^+-dependent component (29%) of lysine transport. However, the majority of the BBM uptake occured through Na^+-independent routes (71%) (See Figure 3.1). Therefore, the present results suggest the presence of both Na^+-dependent and Na^+-independent lysine transporters in the rat intestinal BBM.

When lysine transport in the rat intestine was first characterized in the early 1960's [54], it was determined that there was a system specific for cationic amino acids, system y^+. However, after that initial investigation, many groups have reported the interaction of neutral amino acids with the intestinal BBM dibasic amino acid transport system. Munck and Schultz [118] demonstrated that low concentrations of leucine could stimulate lysine transport and at higher concentrations inhibited lysine transport across BBM of the rabbit intestine. The results of the present sleeve experiments show a considerable degree of inhibition (60%) by L-leucine both in the presence and absence of Na^+ (Figure 3.4). The IC_{50} values are within the range of the apparent K_m estimated for lysine in Figure 3.3, which suggests a common transport system (s) with a high affinity for both dibasic amino acids and L-leucine. Further inhibition of L-leucine at higher concentrations (20 mM) suggests the existence of a second Na^+-independent lysine transport system that interacts which has a low affinity for L-leucine. Additive inhibition experiments in Figure 3.8 suggest this system interacts with L-alanine and L-valine in the presence of Na^+. The inhibition curves from Figures 3.5-3.7 define a third Na^+-independent system for L-lysine which interacts with alanine, valine, and phenylalanine in the presence and absence of Na^+. A fourth component that was identified by the sleeve experiments is a Na^+-dependent system that has $B^{o,+}$ like characteristics of transport. Therefore, these results characterizing BBM lysine transport demonstrate the existence of multiple transport systems in the rat intestinal BBM.

B) Basolateral Membrane Lysine Transport

Lysine transport in the small intestine is known to be a two part process involving transport from the lumen across the BBM into the cell and then across the BLM into the blood. The transport across the BBM is against the concentration gradient and suggestions of the coupling of L-lysine transport to the Na^+ gradient seem logical. However, lysine transport across the BLM has been thought to be facilitative because lysine is moving down its concentration gradient from the inside of the cell to the blood. Earlier studies by Munck suggest that leucine can *trans*-stimulate the exit of lysine into the blood [114,118]. This finding was supported by Cheeseman [24] in *in vivo* studies in the frog, where it was shown that at very low concentrations, leucine in the blood can stimulate the exit of lysine across the BLM. This effect could also be seen when leucine was perfused in the lumen. Both these results suggest the existence of a y^+L exchanger in the rat intestinal BLM. Recent studies using BLM vesicles show lysine transport to be entirely Na^+-independent [26]. Inhibition studies have shown that 50% of BLM lysine transport in vesicles is inhibited by leucine in both the presence and absence of Na^+ (C.I. Cheeseman, unpublished data). The apparent K_m for lysine transport across the BLM is within the 1-2 mM range, which is a about 10 times higher than the affinity of BBM lysine transporters in everted sleeve experiments (Figure 3.3). These results suggest at least two low affinity Na^+-independent lysine transport systems in the rat intestinal BLM: 1) a lysine transport system inhibited by leucine 2) a lysine transporter that is insensitive to leucine.

C) Expression of L-lysine transport systems in *Xenopus* oocytes

The oocyte expression results presented in this thesis demonstrate that isolated stage VI oocytes of *Xenopus laevis* can express lysine transport activity, which is due to the microinjection of purified rat intestinal poly $(A)^+$ mRNA. In such oocytes, exogenous lysine transport (0.2 mM extracellular amino acid, 20°C) reached 400 $pmol \cdot oocyte^{-1} \cdot h^{-1}$ compared with the average typical endogenous transport rate of 85 $pmol \cdot oocyte^{-1} \cdot h^{-1}$ (Figure 3.13). The absolute amount of net expressed flux varied somewhat between experiments, as did endogenous lysine transport, so that pooling data was not possible. This was not unexpected, but it necessitated large experiments on several hundred oocytes prepared at the same time from a single frog; all fluxes in oocytes injected with mRNA were repeated on oocytes injected with water that were also obtained from the same animal. In this way errors of flux measurements were kept extremely low, contributing to more reliable analysis of the characteristics of net expressed flux (Figures 3.11-3.18).

Oocytes are normally prepared by collagenase treatment to render them defolliculated or "denuded". This is particularly important for assaying activity of plasma membrane proteins, because follicle cells possess transport capabilities of their own, which are responsible for supplying solutes to the oocytes *in vivo* [18,171]. It has been suggested that collagenase treatment might cause apparent loss of endogenous transport activity [20], but there was no evidence of this. Endogenous lysine transport in collagenase-treated oocytes was not lower than that in oocytes that had been denuded manually. Therefore, since the collagenase treatment has not reduced endogenous transport, the injection of mRNA was not simply restoring an endogenous flux.

In studies expressing foreign mRNA in oocytes, it was not possible to carry out control experiments which would exclude *a priori* activation of endogenous mRNA or endogenous transporters. Circumstantial evidence suggested that the expressed lysine transport activity was indeed mediated by high affinity rat intestinal BBM transporters. First, the characteristics of the expressed lysine fluxes were clearly different from those of endogenous systems present in the oocytes. Second, the size range of intestinal mRNA encoding expressed lysine transport was consistent with that of other amino acid transporters [5,31,147,165] Also, most importantly, the characteristics of expressed fluxes resembled some of the systems identified in the BBM sleeve experiments.

It has been established in the literature that injection of 25-50 ng of total mRNA is enough to induce maximal expression of heterologous proteins [31,58,66]. The results from the present study were in agreement with this, since maximal lysine transport activity was obtained with the injection of 50 ng of total rat intestinal mRNA (Figure 3.11). When total mRNA is injected into the oocyte, foreign mRNA encoding many different proteins as well as endogenous mRNA compete for the translation machinery [161]. A second parameter that is important is the time required after injection to obtain substantial expression of the protein. In Figure 3.12, maximal lysine transport activity was achieved after 6-7 days in both NaCl and ChCl. This level of expression was maintained for 9 days after injection. Since most batches of eggs remained healthy for one week, flux experiments routinely were carried out 5 days after injection. This time period not only ensured high-level expression and viability of the oocytes, but also maintained a weekly schedule of experiments. Post injection assay times vary for the membrane protein in question and range from 2-3 days up to 7-8 days [31-33,53].

For a long period of time, dibasic amino acid transport was classified under one system, which was Na^+-independent and specific only for dibasic amino acids; classical system y^+. However, many studies have shown the existence of systems for dibasic amino acid transport which are Na^+-dependent [141,174,191]. van Winkle *et al* [173] characterized a system $B^{0,+}$ in mouse blastocysts which is Na^+-dependent and transports both dibasic and some neutral amino acids. In the intestine, Munck and Schultz, Cheeseman, and others have shown there to be an interaction between some neutral amino acids and dibasic amino acids [24,114,116,118,140]. Results from both the sleeve and the expression studies have identified a Na^+-dependent component of lysine transport. As referred to earlier, the Na^+-dependent system in the BBM sleeve experiments has similar characteristics as system $B^{0,+}$. From the oocyte expression experiments in the presence of Na^+, leucine is a potent inhibitor of exogenous lysine transport, capable of abolishing all of the expressed routes with high affinity, albeit slightly lower than arginine. This is not a characteristic of the system designated y^+.

A further interesting observation is that in the absence of Na^+, when only the Na^+-independent routes were assayed, the inhibitory action of leucine was very different. In the experiment in Figure 3.15, leucine was able to inhibit 67% of the Na^+-independent net lysine flux but unable to inhibit the remaining 33%. In previous studies in rat intestinal BBM vesicles, the Na^+-dependence of leucine inhibition was not addressed, presumably because investigators assumed they were dealing with y^+-like mechanisms. However, in this study the results suggest the presence of two Na^+-independent lysine transporters: one of which was inhibited by leucine in the absence of Na^+ with reduced affinity (IC_{50} at 0.83 ± 0.07 mM) and the other is uninhibitable in the absence of Na^+ (Figure 3.15 a,b). In the presence of Na^+, both of the Na^+-independent lysine transporters could be inhibited by leucine with high affinity.

Recently, high-affinity leucine inhibition of lysine transport (1μM) was reported in human red blood cells, allowing the identification of a dibasic amino acid transporter, system y^+L, which is distinct from the y^+ system [39]. Results from both the sleeve and expression studies showed that high-affinity leucine inhibition of high-affinity lysine transport also occurred in the rat intestine. The present work supports red blood cell study which characterizes leucine inhibition as Na^+-dependent [39].

It can be suggested, on the basis of the amino acid inhibition and Na^+-free studies, that three rat intestinal lysine transport activities have been expressed in *Xenopus* oocytes: 1) a Na^+-dependent system that could be inhibited by leucine with high affinity (the Na^+-dependence of this interaction cannot be tested) and that could also probably be inhibited by alanine; 2) a Na^+-independent system which could be inhibited by leucine when Na^+ was present, but this affinity was reduced in the absence of Na^+; 3) a Na^+-independent system that was inhibited by leucine with high affinity but only when Na^+ was present.

These systems expressed in oocytes were probably rat intestinal BBM transport activities. The activities expressed in oocytes are high affinity ($\approx$0.20 mM) and specific for dibasic amino acids and leucine, which is similar to what was observed in the BBM sleeve experiments. In contrast, the BLM lysine transport proteins have an estimated K_m of 1-2 mM, and, as well the interaction of leucine is of relatively low- affinity (C.I. Cheeseman, unpublished data). Experiments were conducted using *Xenopus* oocytes to determined whether total intestinal mRNA expressed lower affinity lysine transport systems. No expression was observed when higher concentrations of lysine (up to 20 mM) were used during transport assays (data not shown). A Na^+-dependent component of lysine transport has been identified in BBM vesicles [174,191] but not in basolateral membrane vesicles (C.I. Cheeseman, unpublished results). In BBM vesicles, there are also several reports of leucine acting as a *cis*-inhibitor of lysine transport with high affinity [114,119], but the Na^+-dependence and possible cation-dependent changes in affinity of the leucine interaction have not been investigated. This was addressed by the expression studies in this thesis and the results suggested the possibility of transport systems similar to $b^{0,+}$ and $B^{o,+}$ existing in the rat intestine. In Table 2.1, specific substrates for these mouse blastocyst systems, BCH and L-valine, inhibited the net lysine flux into oocytes significantly. The net expressed flux was not inhibited by phenylalanine, either in the presence or the absence of Na^+, but was totally abolished by arginine, indicating that there was no significant contribution from low-affinity systems for neutral amino acids such as system L.

Size fractionation of intestinal mRNA yielded information concerning the sizes of the mRNAs coding for intestinal lysine activities. Some expression was found in a fraction ranging from 2.0 to 2.8 Kb, but peak activity of arginine-inhibitable lysine influx was found in size fraction of 1.5-2.25 Kb. This is in the range expected for a membrane spanning protein that functions as a transporter: the Na^+-glucose transporter mRNA transcript is 2.3 Kb [57,58]; liver system L is 2-3 Kb [156]; liver system A is 1.9-2.5

Kb [165].

cDNAs have been isolated recently from rat and rabbit kidney which, when expressed in *Xenopus* oocytes, elicit increases in Na^+-independent neutral and cationic amino acid transport activity [15,167,177]. In contrast to a previously cloned murine fibroblast y^+-type transporter (mCAT family), which has 14 putative transmembrane domains (622 amino acid residues) [74,171], these cDNAs (79% identity in nucleotide sequence) encode 683- and 677- amino acid type II membrane glycoproteins with a single transmembrane regions. The encoded proteins are homologous to α-amylases and α-glycosidases, but have apparently no such enzymatic activity. mRNA for the rat and rabbit proteins, designated D2 and rBAT respectively, was detected only in kidney and intestine through northern blot analysis [16,178,193]. It has also been reported that low-level system y^+L activity is induced by the human fibroblast 4F2hc glycoprotein when expressed in oocytes [14]. The 4F2hc protein, which is structurally related to D2/rBAT, is ubiquitous [14] but its occurrence in the intestine was unknown until this present study. In this thesis, diagnostic PCR revealed that both D2 and 4F2hc are found in the rat jejunum (Figure 2.2). Antisense-hybrid depletion experiments were performed for both D2 and 4F2hc. The results from Figure 3.18 indicate that both D2 and 4F2hc contributed to the lysine transport activity expressed in oocytes injected with total jejunal mRNA. Interestingly, the addition of both 4F2hc and D2 antisense oligonucleotides did not cause an additive reduction in the lysine flux. The ratio of antisense oligonucleotide to intestinal mRNA was 4:1 (*w/w*). Higher ratios were not tested because investigators have suggested that there can be non-specific inhibitory effects if too much antisense is used [43,112]. Use of sense cRNA provided a control for possible non-specific inhibitory effects. Even though a portion of the net expressed lysine flux maybe due to D2 and/or 4F2hc, the majority ($\approx$60%) remained unaffected by the addition of antisense oliognucleotides. Also, the Na^+-dependent component of expressed lysine transport activity cannot be due to D2/4F2 because both D2 and 4F2hc induced Na^+-independent lysine transport activity. The Na^+-independent transport of lysine, induced by D2 is inhibited completely by phenylalanine [178,179] whereas the expressed lysine flux observed in my experiments was uninhibited by phenylalanine at 1 mM (Figure 3.17). Since, the majority of the lysine transport activity expressed in oocytes injected with intestinal mRNA appeared to be unrelated to 4F2hc and D2 a size fractionated cDNA library was created and efforts were directed towards cloning proteins responsible for the intestinal lysine transport activity.

D) Rat intestinal 4F2hc - Transporter or Regulator

The cloning of a cDNA encoding rat intestinal 4F2hc began with the screening a cDNA library that had a mean insert size of 2.4 Kb (1.6-3.0Kb). This was overlapping with, but slightly higher than, the size fraction of 1.5-2.25 Kb found to express peak lysine transport activity (See Figure 3.19,3.20). The latter rat intestinal mRNA size fraction was provided to Dr. John Elliot, Department of Microbiology and Infectious Diseases, University of Alberta to make a cDNA library that contained between one to two million primary recombinants. Injection into oocytes of cRNA made from this total library resulted in only marginal expression of lysine transport activity (data not shown). At the same time a smaller rat intestinal cDNA library was created by Dr. Q.Q. Huang in this laboratory from a 1.60 -3.0 Kb mRNA fraction for the screening of nucleoside transport activity [194]. The cRNA from this cDNA library induced a 3-4 fold increase in lysine uptake into oocytes compared to the water-injected controls (See Figure 3.21). Four of the pools tested were positive for lysine transport activity and the level of expression was increased (up to 9-10 fold above background) compared with that seen for the total library (See Figure 3.22). However, all four pools were positive for 4F2hc, which suggested that this high lysine transport activity might, at least in part, due to

4F2hc. Subdivision of pool #47 led to a decrease in activity (See Figures 3.23,3.24,3.26). During expression cloning when the cDNA library is divided into smaller and smaller pools, the activity of that positive cDNA in these pools normally becomes more pronounced. There are several possible reasons why, in the present case, the activity decreased instead of increased. First, the cDNA contributing to major part of the expressed lysine transport activity may have been lost during the transfer of individual bacterial colonies from the master plate to the wells of the microtitre plates. A second possibility is that two different cDNAs were required to give maximum lysine transport activity. This would be the case, for example, if the functional transporter complex comprised a heterodimer of two different subunits or like the homo-oligomeric functional potassium channel that contains subunits, each with a single transmembrane region [162]. I attempted to test for this possibility by taking cDNA from the positive row of pool #47 and mixing it with cDNA from each of the other rows from this pool. I was unable to demonstrate any further increase in lysine transport activity (data not shown).

The characterization of the lysine transport activity encoded by the rat intestinal 4F2hc clone began with the determination of the K_m for lysine transport. The K_m $\approx$0.04-0.05 mM in both the presence and in the absence of Na^+ (See Figure 3.31). Therefore, the apparent affinity of the 4F2-induced lysine influx was about 4 times higher than that estimated during the expression studies using total rat intestinal mRNA (i.e $\approx$0.20 mM - Figure 3.9). This was consistent with the antisense hybrid-depletion experiment shown in Figure 3.18 which suggested that 4F2hc accounted for only part of the lysine transport activity encoded by rat jejunal mRNA. In Figure 3.32, the majority of the exogenous lysine influx induced by the 4F2hc clone was Na^+-independent. Removal of Na^+ slightly reduced the expressed lysine transport activity. Therefore, the results suggested that 4F2hc induced a single Na^+-independent lysine transport activity when injected into oocytes. To test whether 4F2hc was native to the oocyte, mRNA was extracted from oocytes, reverse transcribed into cDNA and tested by diagnostic PCR with the human 4F2hc primers. Rat intestinal mRNA was used as a positive control and no PCR product was produced from the oocyte cDNA (data not present). Therefore, the mRNA for the 4F2hc protein did not appear to occur naturally in the oocyte.

Competition experiments with neutral amino acids helped determine amino acid specificity of the 4F2hc-induced lysine flux. In Figure 3.32, leucine inhibition of lysine influx was clearly Na^+-dependent, which resembles the characteristics of the y^+L system, first identified in human red blood cells [39]. The other neutral amino acids have modest inhibitory effects in both the presence and absence of Na^+, with inhibition being somewhat greater in Na^+ containing medium. This inhibition is not characteristic of the $b^{0,+}$ system, as Na^+-dependent inhibition of lysine transport was not reported [172]. Inhibition by phenylalanine of lysine transport was not evident in the expression studies using total intestinal mRNA. Thus, it appeared even though this 4F2hc induced lysine transport interacts with a number of neutral amino acids it does not appear to be *via* either $B^{0,+}$ or $b^{0,+}$.

Since leucine was by far the best inhibitor of 4F2-induced lysine transport, the question arose as to whether it was actually being transported by the same system. Efflux experiments (See Figure 3.33,3.34) demonstrated that expression of 4F2hc resulted in the influx of leucine in exchange for the efflux of lysine. Moreover, stimulation of efflux occured only in the presence of Na^+. Similar interactions involving leucine and lysine were seen in rat BLM vesicle experiments by Munck and Schultz [118] and Cheeeseman *et al* [88] and anuran *in vivo* experiments by Cheeseman [24]. The transport activity also resembles, in part, what was found in the BBM sleeve experiments and it remains to be determined where this 4F2hc protein is located; BBM or BLM.

Since the amino acid sequence of rat intestinal 4F2hc is highly homologous to both the human and mouse isoforms (See Figures 3.28,3.29), the characteristics of the transport induced by these proteins should be similar. Comparing the studies done on the mouse and human 4F2hc proteins, a Na^+-independent uptake of dibasic amino acids is observed and the inhibition profile matches that shown for rat intestinal 4F2hc in this study [14,27]. 4F2hc belongs to a family of type II glycoproteins which includes the rBAT protein. The similarity between 4F2hc and rBAT amino acids sequences is 79% which suggests that these two proteins could be related (Figure 4.1). A recent study [19] of rBAT in cystinuric patients revealed several amino acid mutations within a highly conserved region of the protein. Oocyte expression of one of the most frequent mutations (M467T) resulted in an 80% reduction of the induced amino acid fluxes. Comparison of this region of rBAT with 4F2hc shows a very high degree of conservation which suggests a similar role for 4F2 in the small intestine to that for rBAT in the kidney. However, 4F2hc does not induce cystine transport [14,179], so while this protein may not play a role in cystinuria it could well be implicated in the control of the transport of other amino acids in the small intestine, for example, lysine. If this assumption is correct then it should be possible to reduce or abolish the induced lysine flux by mutating the same region within 4F2hc. Therefore, it will be important to test the effect of substituting these potentially critical amino acid residues in the oocyte expression system and determine the effect on the induced lysine transport activity.

Many transport proteins (eg., the equilibrative glucose transporters [13,49,74] and Na^+/glucose co-transporter [58], several nucleoside transporters [69,71], and the glutamate transporter family [69,71]) have 12-14 transmembrane spanning regions. In contrast, the amino acid sequences of mouse and human 4F2hc predicts a single transmembrane region. The hydropathy plot in Figure 3.30 predicts that rat intestinal 4F2hc also contains a single transmembrane spanning region. The structure of the protein is therefore more consistent with a regulator/modulator function than a transport function.

```
        1                                                  50
111696  MAEDKSKRDS IEMSMKGCQT NNGFVHNEDI LEQTPDPGSS TDNLKHSTRG
m90096  MAEEGSKRDS IKMNMKGCQT NNGFVQNEDI PELDLDPGSS EH........
r4f2hc  ......MSQD TEVDMKDVEL N.ELEPEKQP MNAADGAAAG EKNGLVKIKV

        51                                                100
111696  ILGSQEPDFK GVQPYAGMPK EVLFQFSG.Q ARYRIPREIL FWLTVASVLV
m90096  ILGPEEPNAK NIQPYAGMPK EVLFQFSG.Q ARYRVPREIL FWLVVVSVLV
r4f2hc  AEDEAEAGVK ....FTGLSK EELLKVAGSP GWVRTRWALL LLFWLGW.LG

        101                                               150
111696  LIAATIAIIA LSPKCLD... ..WWQEGPMY QIYPRSFKDS NKDGNGDLKG
m90096  LIGATIAIIA ISPKCLD... ..WWQAGPMY QIYPRSFKDS DKDGNGDLKG
r4f2hc  MLAGAVVIIV RAPRCRELPV QRWWHKGALY RI..GDLQAF VGPEARGIAG

        151                                    *          200
111696  IQDKLDYITA LNIKTVWITS FYKSSLKDFR YGVEDFREVD PIFGTMEDFE
m90096  IQDKLDYITT LNIKTIWITS FYKSSLRDFR YGVEDFREID PIFGTMEDFE
r4f2hc  LKNHLEYLST LKVKGLVLGP IHKNQKDEV. .NETDLKQID PDLGSQEDFK

        201                                               250
111696  NLVAAIHDKG LKLIIDFIPN HTSDKHIWFQ LSRTRTGKYT DYYIWHDCTH
m90096  NLLAAIHDKG LKLIIDFIPN HTSDKHPWFQ LSRTRTGKYT DYYIWHDCAH
r4f2hc  DLLQSAKKKS IHIILDLTPN YKGQ...... .......... ..........

        251                                               300
111696  ENGKTIPPNN WLSVYGNSSW HFDEVRNQCY FHQFMKEQPD LNFRNPDVQE
m90096  ENGITTPPNN WLSVYGNSSW HFDEVRNQCY FHQFLKEQPD LNFRNPDVQE
r4f2hc  ........NA W......... .......... ...FLPPQAD I......VAT

        301                                               350
111696  EIKEILRFWL TKGVDGFSLD AVKFLLEAKH LRDEIQVNKT QIPDTVTQYS
m90096  EIKEIMQFWL TKGVDGFSFN AVKFLLEAMH LRNEIQVNAS QIPDTVTRYS
r4f2hc  KMKEALSSWL QDGVDGFQVR DVGKLANASL YLAEWQNITK NFSEDRLLIA

        351                                               400
111696  ELYHDFTTTQ VGMHDIVRSF RQTMDQYSTE PGRYRFMGTE AYAESIDRTV
m90096  ELYHDFTTTQ EGMHDLVRSF RQTMDKYSRE PGRYRFLGTE AYAESIDRTM
r4f2hc  G......TAS SDLQQIVNIL ESTSDLLLTS S....YLSQP VFTG......

        401                                               450
111696  MYYGLPFIQE ADFPFNNYLS MLDTVSGNSV YEVITSWMEN MPEGKWPNWM
m90096  RYYGLSFIQE ADFPFNNYFT TLDTLSGNTV YEVITAWMEN MPEGKWPNWM
r4f2hc  ........EH AELLVIKYL. .......... .........N ATGSRWCSWS

        451                     *                         500
111696  IGGPDSSRLT SRLGNQYVNV MNMLLFTLPG TPITYYGEEI GMGNIVAANL
m90096  TGGPDITRLT SRLGNQYVNI MNMLLFTLPG TPITYYGEEI GMGNILATNL
r4f2hc  VSQAGL..LT SFIPDQFLRL YQLLLFTLPG TPVFSYGDEL GLQAVALPGQ

        501                                               550
111696  NESYDINTLR SKSPMQWDNS SNAGFSEASN TWLPTNSDYH TVNVDVQKTQ
m90096  NESYDVNTLL SKSPMQWDNS SNAGFSEGNH TWLPTSSDYH TVNVDVQKTQ
r4f2hc  P........M EAPFMLWNES SN......SQ TSSPVSLN.. .MTVKGQNED

        551                                               600
111696  PRSALKLYQD LSLLHANELL LNRGWFCHLR NDSHYVVYTR ELDGIDRIFI
m90096  PTSALKLYQA LSLLHANELL LSRGWFCLLR NDSRVLVYTR ELDGIDRVFI
r4f2hc  PGSLLTQFRR LSDLRGKERS LLHGDFDALS SSSGLFSYVR HWDQNER.YL

        601                   *                           650
111696  VVLNFGESTL LNLHNMISGL PAKMRIRLST NSADKGSKVD TSGIFLDKGE
m90096  VVLNFGESTL LNLQEMISGL PVRLSIKLST NSASTGSQVD TRGIFLERGE
r4f2hc  VVLNFQDVG. LSARVGASNL PAGISLPASA NLL.....LS TDSTRLNREE

        651    *                                  691
111696  GLIFEHNTKN LLHRQTAFRD RCFVSNRACY SSVLNILYTS C
m90096  GVLLEHKMKN LLHRQTAFRD RCFISSRACY SSALDILYSS C
r4f2hc  GTSLSLENLS LKPDEGLLLQ FPFVA..... .......... .
```

Figure 4.1 Alignment of the amino acid sequences of human rBAT (- 111696 -); rBAT-1 from rabbit (- m90096 -); rat intestinal 4F2hc (- r4F2hc -). * refers to the amino acid residues in human rBAT that are mutated in cystinuric patients: arginine 181; methionine 467; proline 615; threonine 652.

E) Future Studies

Cloning of Other Lysine Transporter Proteins

The sleeve and oocyte expression experiments described in this thesis indicate the presence of multiple lysine transport activities in the rat jejunal enterocytes, the majority of which are unlikely to be related to D2 or 4F2hc glycoproteins. Also, if D2 and 4F2 function as regulators, then corresponding transport proteins must be also present in the intestine. Two cDNA libraries have been prepared from rat jejunum. As a first step, both should be rescreened for lysine transport activity in an attempt to isolate transport-related cDNAs other than D2 or 4F2 and resolve the anomalous finding described in Section 3 (Cloning studies) of RESULTS where subcloning of pool #47 lead to a decrease rather than increase in expressed lysine transport activity.

Location and further characterization of rat intestinal 4F2hc

With the cloning of rat intestinal 4F2hc and the results obtained from the characterization experiments, there are two possibilities concerning its physiological role. If in fact 4F2hc is an actual transport protein then its role could be defined as moving lysine from the lumen of the intestine across the BBM into the cell or from the cell interior across the BLM to the blood. However, the location of this protein in the enterocyte is still unsolved. Immunocytochemistry studies are needed to determine whether 4F2hc is expressed in the BBM, BLM, or some internal compartment of the cell. rBAT, the protein which is closely related to 4F2hc, has been located in the BBM of the kidney proximal tubule and intestine [70,133] If 4F2hc is found to be located in the BLM it could be involved in either LPI or dibasic aminoaciduria. It will be informative to establish whether or not mutations equivalent to those occuring in rBAT in cystinuria affect expression of 4F2hc-mediated lysine transport in oocytes. Rat intestinal 4F2hc should be further characterized in other expression systems such as yeast or mammalian cell lines. If it is a regulatory protein, does it regulate similar system(s) in these host cells? To answer the question of whether 4F2hc is a regulator or a functional transporter, it will be necessary to functionally reconstitute recombinant 4F2hc protein into an artificial membrane where other transport proteins are not present. If carrier-mediated lysine transport activity is induced, it could be concluded that 4F2hc is indeed a transport protein.

Cloning and expression of the 4F2hc light chain

The 4F2hc protein that was cloned in this study is referred to as the heavy chain antigen [136], there being a corresponding 35 Kd non-glycosylated light chain which is thought to be connected to the heavy chain through disulfide bridges [129]. One of the two cysteine residues found in rat intestinal 4F2hc (see Figure 3.27) is also conserved in D2. This raises the questions of whether 4F2hc light chain is also involved in dibasic amino acid transport and in what capacity? cDNA cloning and functional expression of the 4F2 light chain in oocytes, both alone and in combination with the heavy chain, should be an important priority for future research regarding 4F2hc.

F) Summary

Transport studies using everted sleeves from rat jejunum and expression experiments in *Xenopus* oocytes injected with rat jejunal mRNA have identified multiple lysine transport activities which have common atypical characteristic of being inhibited, with high-affinity, by the neutral amino acid L-leucine. cDNA cloning by functional expression of lysine transport activity in *Xenopus* oocytes resulted in the isolation of a cDNA encoding a 4F2hc type II membrane glycoprotein. This protein is homologous to D2, mutations of which result in cystinuria, a human genetic disease causing defective intestinal and renal transport of lysine, other dibasic amino acids, and cystine.

Expression of 4F2hc in *Xenopus* oocytes induced a novel bifunctional amino acid transport activity (y^+L) in which high affinity Na^+-independent transport of lysine and high affinity Na^+-dependent transport of leucine are probably mediated by the same transport protein. It is proposed that 4F2hc may have a physiological role in lysine/leucine transport in intestine and perhaps, as well, other tissues.

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APPENDIX

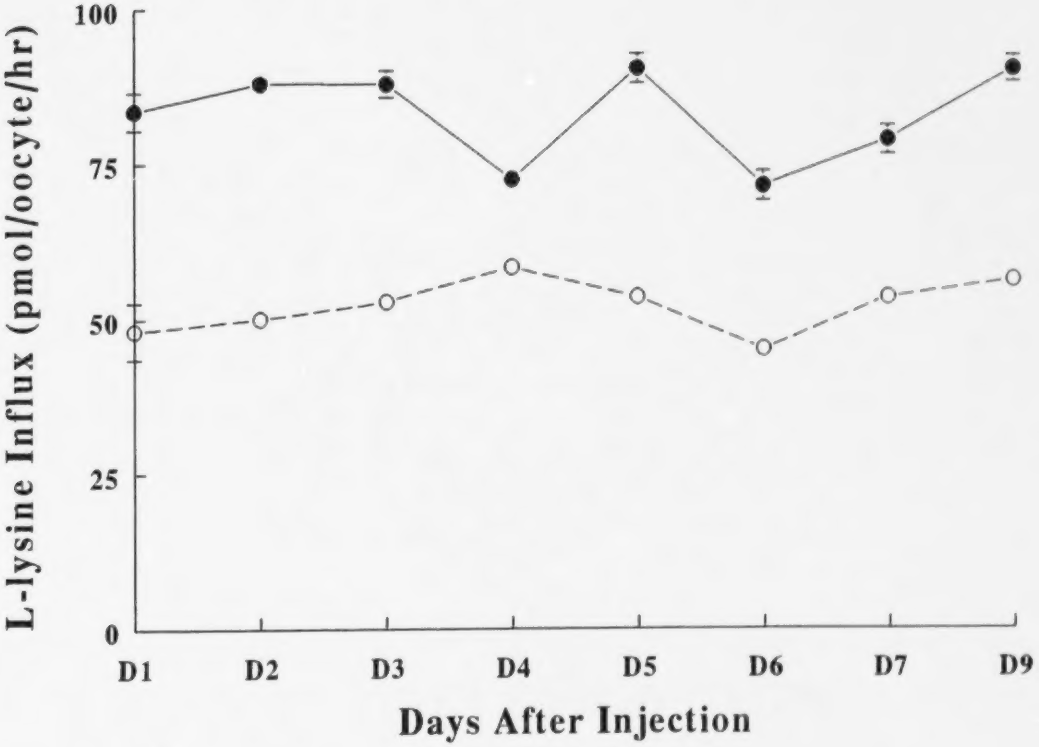

Figure 5.1 Influx of L-lysine (0.2 mM) into isolate oocytes of *Xenopus laevis* as a function of post-injection period. Results shown are for uptake after 2-9 days following injection of water (Control). Fluxes were performed in NaCl TB (closed circles) and ChCl TB (open circles). Each data point is the mean uptake into ten individual oocytes injected with water. Error bars are the SE of the mean. All oocytes came from one frog.

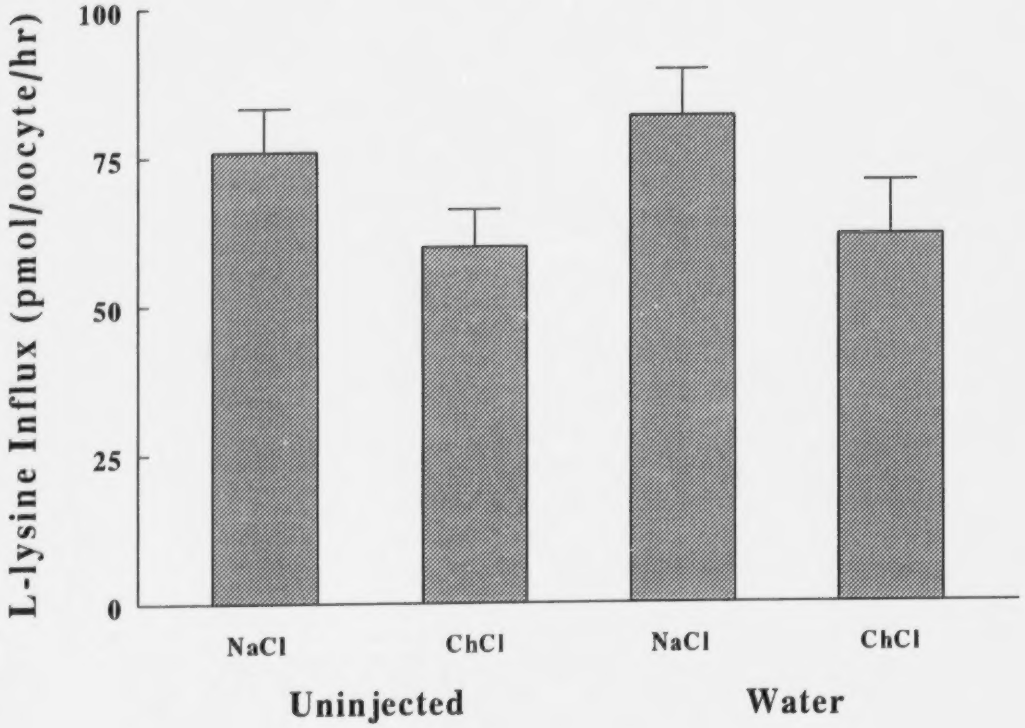

Figure 5.2 Influx of L-lysine (0.2 mM) into isolated oocytes of *Xenopus laevis* that were either not injected or injected with water. Fluxes were performed in either NaCl or ChCl TB. Each data point is the mean of uptake into ten uninjected individual oocytes or ten individual oocytes injected with water. Errors are the SE of the mean. All oocytes came from one frog.